BULLETIN
OF
THE JOHNS HOPKINS HOSPITAL

(THE PUBLICATION OF THE MEDICAL SCHOOL AND HOSPITAL)

(SUPPORTED BY THE DE LAMAR FUND OF THE JOHNS HOPKINS UNIVERSITY)

EDITORIAL BOARD

Managing Editor, E. Cowles Andrus

Associate Managing Editor, John A. Luetscher, Jr.

Charles R. Austrian
W. Halsey Barker
Kenneth C. Blanchard
Edward M. Hanrahan
John Eager Howard
Arnold R. Rich

VOLUME LXXXI

BALTIMORE
THE JOHNS HOPKINS PRESS
1947

COPYRIGHT, 1947
BY THE JOHNS HOPKINS PRESS

HOSPITAL LIBRARY OCT 13 1947

VOLUME 81 NUMBER 1

BULLETIN of the JOHNS HOPKINS HOSPITAL

THE PUBLICATION OF THE MEDICAL SCHOOL AND HOSPITAL
SUPPORTED BY THE DE LAMAR FUND OF THE JOHNS HOPKINS UNIVERSITY

July, 1947

Studies on the Intravascular Thromboplastic Effect of Tissue Suspensions in Mice. I. The Reaction of Mice to Intravenous Injections of a Sedimentable Tissue Component. LEWIS THOMAS 1

Studies on the Intravascular Thromboplastic Effect of Tissue Suspensions in Mice. II. A Factor in Normal Rabbit Serum Which Inhibits the Thromboplastic Effect of the Sedimentable Tissue Component. LEWIS THOMAS 26

Polymyxin: A New Chemotherapeutic Agent. P. G. STANSLY, R. G. SHEPHERD AND H. J. WHITE 43

5-Halo-2-Thenyl Derivatives of N,N-Dimethyl-N′-2-Pyridyl-Ethylenediamine as Antihistaminics. J. T. LITCHFIELD JR., MAXINE R. ADAMS, LOUISE GODDARD, MARION S. JAEGER AND LILLIAN ALONSO 55

Podophyllotoxin. MAURICE SULLIVAN AND KENNETH BLANCHARD 65

Proceedings of the Meetings of the Johns Hopkins Medical Society 68

PUBLISHED MONTHLY

THE JOHNS HOPKINS PRESS, Baltimore-18, Maryland

Made in United States of America

COPYRIGHT, 1947, BY THE JOHNS HOPKINS PRESS

BULLETIN
OF
THE JOHNS HOPKINS HOSPITAL

MANAGING EDITOR, E. COWLES ANDRUS

ASSOCIATE MANAGING EDITOR, JOHN A. LUETSCHER, JR.

EDITORIAL BOARD

Charles R. Austrian — James Bordley, III
W. Halsey Barker — John Eager Howard
Arnold R. Rich

All correspondence relating to the publication of papers should be addressed to The Editor, Bulletin of The Johns Hopkins Hospital, Baltimore-5, Md.

Books for review should be sent to The Editor at the above address.

Twenty-five reprints, without covers, of articles will be furnished to contributors free of cost. An order slip for additional reprints, with a table showing cost, is sent with proof.

Correspondence concerning business matters should be sent to The Johns Hopkins Press, Baltimore-18, Maryland, U. S. A.

This periodical is issued monthly. Two volumes a year are issued, each consisting of approximately 400 pages, and beginning in January and July, respectively.

Subscription price. In the United States and its possessions $6.00 per year. In Canada $6.25 per year; in foreign countries $6.50 per year. Prices for back volumes and back single numbers on application. Single numbers of the current volume, when available, $.75. Prices are net, postpaid.

New subscriptions and renewals are entered to begin with the first issue of the current volume. Should any issue of the current volume be out of print at the time the subscription order is received, the pro rata value of such numbers will be credited to the next volume, and the renewal invoice therefor adjusted accordingly.

Subscriptions should be renewed promptly. To avoid a break in your series, subscriptions should be renewed promptly. The publisher cannot guarantee to supply back issues on belated renewals.

Subscriptions, new or renewal, may be sent to the Publishers or ordered through your agent.

Subscriptions in the British Empire except Canada may be sent to: Baillière, Tindall & Cox, 8 Henrietta St., Covent Garden, London, W.C. 2

THE JOHNS HOPKINS PRESS
BALTIMORE-18, MARYLAND, U. S. A.

Entered as second-class matter at the Baltimore, Md., Postoffice. Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917. Authorized on July 3, 1918.

Hospital
of
Dr. Carl V. Weller

STUDIES ON THE INTRAVASCULAR THROMBOPLASTIC EFFECT OF TISSUE SUSPENSIONS IN MICE

I. The Reaction of Mice to Intravenous Injections of a Sedimentable Tissue Component

LEWIS THOMAS.

From the Department of Pediatrics, Johns Hopkins University Medical School, and The Harriet Lane Home for Invalid Children, Baltimore, Maryland

The property of blood to maintain its normal fluid state as it circulates through the vascular compartment is generally assumed to be the result of an equilibrium system, in which opposing forces, those which initiate and those which inhibit coagulation, are kept constantly in balance. The nature of the factors which participate in the maintenance of this equilibrium, and the mechanisms which may displace the balance to one direction or the other, are still subjects for speculation. The available methods for studying the physical state of the blood *in vitro* are limited not only by the complexity of the coagulation mechanism itself, but also by the probable absence of unknown factors whose participation may be crucial. In order to investigate the forces involved in the equilibrium, it is desirable to have methods which deal with the living animal and which permit the observation of experimentally induced deviations from the normal, *in vivo*.

It has long been known that the intravenous injection of tissue extracts in animals may cause severe toxic reactions, which are associated with the widespread formation of intravascular thrombi (1, 2), presumably due to the presence of thromboplastic substances in the tissue extracts.

During the course of an unrelated study of neurotropic viruses in this laboratory, it was observed that the intravenous injection of dilute suspensions of mouse brain tissue produced, with great regularity, a series of characteristic and easily recognizable symptoms in mice. For a period of approximately 20 seconds after injection, the animals appeared to be entirely well. They then suddenly exhibited grossly incoordinated movements, followed within a few seconds by generalized clonic convulsions and coma. The onset of these symptoms was associated with an abrupt change in the appearance of

L.C

circulating blood in the smaller vessels of the ear and mesentery, as seen by direct microscopic examination. The blood slowed sharply, and, in many vessels, came to a complete standstill, with the formation of irregular masses of apparently aggregated blood cells. At the same time, the coagulation time of the blood was found to be markedly shortened. These changes were, in most instances, transitory; within a period of some seconds the blood began to move through the vessels again, and soon became normal in appearance in vessels which had apparently been occluded a few seconds earlier. The clotting time, several minutes later, was found to be prolonged considerably beyond normal.

It was then found that when mice were given a preliminary injection of small amounts of brain tissue suspension, insufficient to cause symptoms, they became resistant within a few minutes to the toxic effect of much larger doses of the same material. This state of resistance was associated with a prolongation of coagulation time similar to that which occurred following the injection of larger, convulsion-producing doses.

The neurological symptoms in mice, and the observed changes in the appearance of circulating blood in the peripheral vessels, were completely prevented by the administration of heparin.

It is the purpose of the present paper to describe these events in detail, and to present certain related observations which bear upon the physiological disturbances involved.

METHODS

Two types of tissue suspensions were used for the injection of mice in the experiments to be reported. These were: a) crude suspensions, which consisted of ordinary saline extracts of ground tissue, centrifuged at low speed to remove gross tissue particles, and b) saline suspensions of the tissue component which was sedimentable by centrifugation of crude suspensions at 12,000 R.P.M.

A variety of tissues was employed in the experiments, including brain, lung, heart, liver, spleen, kidney and muscle, from mouse, rabbit and human sources. Fresh tissue, or specimens which had previously been stored in the frozen state at $-60°$C., were weighed and then ground in a mortar with an abrasive. Physiological saline solution was added to the ground tissue in a volume sufficient to

produce a 10 per cent suspension. The suspension was centrifuged at 2,000 R.P.M. for 10 minutes. The supernatant fluid will be referred to in this paper as the "crude suspension".

The crude suspension was centrifuged at 12,000 R.P.M. for 30 minutes on a motor-driven angle centrifuge, in an environment of 4°C. The supernatant fluid was discarded, and the pellet of sediment was resuspended in saline solution equal to the original volume. This suspension will be referred to as "the sedimentable tissue component".

For most of the experiments to be reported, tissue suspensions of either type were prepared in quantities of 100 ml. or more, distributed in 8 ml. amounts in lusteroid tubes, and stored at −60°C. Tissue suspensions stored in this manner showed little or no deviation from their original activity when injected into mice, after storage for 4 weeks.

Mice were injected intravenously, in the tail vein, with 0.2 ml. of the material under test. This amount was administered within two seconds. The animals were then observed carefully for a period of at least two minutes. It was found that when symptoms occurred at all, they appeared in almost every instance during the first minute after injection. Reactions were regarded as positive when mice exhibited gross ataxia, falling, and convulsive movements of the extremities. At least two mice were used for each material tested, and when endpoints were being determined in titrations of tissue suspensions four or five mice were injected.

Some variation in susceptibility to the effect of tissue suspensions was encountered when mice of different sizes were tested. Large mice were sometimes unaffected by doses which produced ataxia and convulsions in smaller mice. For this reason, mice weighing between 20 and 25 grams were used for all of the experiments to be reported. The mice were white swiss stock, obtained from a single breeder.

In the text and tables to follow, the dilutions of tissue suspension will be referred to on the basis of the original weight of whole tissue. For example, the 10 per cent suspension will be designated as the 1–10 dilution, and two-fold dilutions of this as 1–20, etc.

DESCRIPTION OF THE REACTION IN MICE

The following description of symptoms applies not only to mice injected with brain tissue suspensions, but also to animals receiving

suspensions of the sedimentable component of a variety of other tissues, as will be shown in a subsequent section.

For a period of between 15 seconds and one minute following the injection, the mice appeared to be unaffected and ran about in a normal manner. At the end of this period, they suddenly stopped in their tracks and began to weave their heads from side to side. After a few seconds, they exhibited incoordinated, grossly ataxic running movements and usually fell to one side. Then, in most instances, they developed violent convulsive movements of the extremities, usually lasting for less than a minute but sometimes recurring at intervals over a period of several minutes. Following the convulsions, the animals lay quietly on their sides or backs, completely immobile except for respirations. Although they appeared to be deeply comatose at this time, many of the mice would undergo a new series of convulsive movements if painful stimuli were applied to the feet or tail. They remained motionless for several minutes, and some of the animals, especially those which received concentrated doses of tissue suspension, died at this time. The majority, however, after about five minutes began to make slow clumsy movements, and after ten minutes were usually on their feet again. At this time, it was not uncommon to see animals with complete paralysis of both hind legs, or with constant involuntary turning or rolling movements. Gradually, after about fifteen more minutes, these symptoms disappeared, and at the end of an hour the mice seemed to be entirely normal.

Variations from the above typical reaction were occasionally observed. When large doses of tissue suspension were injected, the interval preceding the onset of symptoms was shortened and some of the mice underwent a brief, severe convulsion and died within a few seconds, without exhibiting other neurological symptoms. On the other hand, when the suspensions were diluted almost to the limit of their toxic effect, the interval preceding the symptoms was prolonged and the animals showed milder symptoms of ataxia and weakness, with a few brief convulsive movements of the extremities. These mice often recovered within a few seconds.

The constancy with which the reaction could be elicited in large groups of mice, with individual samples of tissue suspension, is illustrated by the following experiment. Two-fold serial dilutions of

two separate suspensions of mouse brain tissue, extending from 1–10 to 1–640, were prepared in physiological saline. Groups of 12 mice were injected intravenously with 0.2 ml. of each dilution, and the reactions were recorded. The results are shown in Table I. It will be seen that with only two exceptions all of the mice in each group reacted similarly to the injected suspensions. The exceptions occurred in the groups which received dilutions corresponding to the endpoint in each titration. The first suspension had its endpoint at the dilution of 1–160, and reactions occurred in eleven of the twelve mice in this group. With the second suspension, all of the twelve mice receiving the 1–80 dilution showed reactions, while one of the group injected with the next higher dilution had a reaction. Each

TABLE I

Occurrence of Ataxia and Convulsions in Groups of Mice Injected with Varying Dilutions of Mouse Brain Tissue Suspensions

TISSUE SUSPENSION	DILUTION OF SUSPENSION*						
	1-10	1-20	1-40	1-80	1-160	1-320	1-640
1	12/12†	12/12	12/12	12/12	11/12	0/12	0/12
2	12/12	12/12	12/12	12/12	1/12	0/12	0/12

* Dilution figures are based on the original weight of tissues.

† Numerator refers to number of mice exhibiting reaction of ataxia and convulsions. Denominator refers to number of mice tested in each group.

of the other dilutions of the suspensions produced uniform results in all mice. These findings indicated that the method of titrating suspensions by two-fold dilution yielded reactions which were sufficiently reproducible to permit comparisons to be made between the activity of different suspensions.

It was of interest to determine how closely the length of the time interval preceding symptoms could be correlated with the concentration of tissue suspension. Two-fold dilutions of a 10 per cent suspension of mouse brain tissue were prepared in physiological saline, and 0.2 ml. of each dilution were injected into mice. The time between the injection and the first symptoms was determined with a stop-watch. The results are shown in Table II. A considerable degree of correlation existed between the concentration of tissue suspension and the

length of the interval required for the appearance of symptoms. The average interval in mice receiving the 1–10 dilution was approximately 19 seconds, compared with 26 seconds following the 1–20 dilution, 33 seconds with the 1–40 dilution, and 58 seconds with the 1–80 dilution.

Reactions were not produced when mice were injected by any route other than intravenous. Large doses of concentrated tissue suspension caused no symptoms when administered subcutaneously and intraperitoneally. Intracerebral injection produced, in some mice, immediate neurological symptoms accountable to local injury of the

TABLE II

Effect of Dosage of Mouse Brain Tissue Suspensions on the Time Interval Preceding the Reaction in Mice

DILUTION OF SUSPENSION	NUMBER OF MICE	TIME INTERVALS IN INDIVIDUAL MICE	AVERAGE TIME INTERVAL
1–10*	6	20″, 20″, 20″, 19″, 18″, 20″†	19.5″
1–20	5	26″, 27″, 25″, 27″, 28″	26.6″
1–40	7	35″, 30″, 30″, 45″, 40″, 35″, 20″	33.5″
1–80	5	55″, 60″, 60″, 70″, 45″	58″

* Figures indicating dilution are based on the original weight of brain tissue.

†Numbers refer to the interval between the time of injection and the appearance of symptoms, expressed in seconds.

brain, but the symptoms did not resemble the reaction following intravenous inoculation.

The Effect of Anticoagulant Drugs

It seemed probable that the symptoms in the injected mice were due to the thromboplastic action of tissue suspensions within the circulating blood, and it was therefore of interest to study the effect on the reaction of two anti-coagulant drugs, heparin and congo red.

A commercially prepared solution of heparin, containing 1.0 mg. of heparin in 10 ml., was diluted serially in physiological saline, and portions of each dilution were mixed with equal volumes of a 10 per cent suspension of mouse brain tissue. Each mixture was then tested by the intravenous injection of 0.2 ml. into four mice. The results are indicated in Table III. It was found that heparin, in amounts as

small as 0.004 mg. per mouse, afforded complete protection against the toxic effect of mouse brain tissue suspension.

The protective action of heparin could also be demonstrated when small amounts of this drug were injected intravenously, several minutes before the injection of tissue suspensions. Moreover, the effect was demonstrable when heparin was injected 15 to 20 seconds after the suspensions, provided that it was given before the onset of symptoms. When the injection of heparin was delayed until after the appearance

TABLE III

Protective Action of Intravenous Heparin and Congo Red Against Toxic Effect of Mouse Brain Tissue Suspensions in Mice

DRUG	DOSE	REACTION*
	mg.	
Heparin	0.25†	0
	0.025	0
	0.004	0
	0.002	+
	0	+
Congo Red	5.0‡	0
	1.0	+
	0	+

* In this and the following tables the reactions of injected mice are expressed by the symbols: + = ataxia and convulsions following injection, 0 = no symptoms.

† Indicates amount of heparin contained in 0.2 ml. of tissue suspension injected.

‡ Indicates amount of congo red injected 5 minutes before tissue suspension.

of ataxia and convulsions it was entirely without effect, and animals which had begun to exhibit symptoms showed no change in their subsequent course.

It has been reported that congo red, when given in large doses, renders the blood of animals incoagulable, although smaller doses may actually increase the coagulability of the blood (3). The mechanism underlying these effects is not known. Similar changes in coagulability were observed in this laboratory, following the intravenous injection of varying quantities of congo red. The injection of 1.0 mg., in a 1 per cent solution, resulted in reductions of the clotting time in mice to one minute or less as compared with the normal clotting time

of 3 to 5 minutes. In contrast, 5.0 mg. of the same material caused a prolongation of clotting time to 2 hours or longer.

Four mice were given intravenous injections of 1.0 mg. of congo red, in a 1 per cent solution, and four other mice received 5.0 mg. Five minutes later, each animal was tested by the intravenous injection of brain tissue suspension, in a dilution of 1–20, and at the same time four untreated mice were similarly tested. The results are shown in Table III. It will be seen that each of the animals receiving 1.0 mg. of congo red exhibited ataxia and convulsions following the injection of tissue suspension. In contrast, the mice which were given 5.0 mg. of congo red showed no symptoms. It was thus evident that doses of this drug which were sufficient to produce an anticoagulant effect also provided protection against the toxic action of tissue suspensions.

The Appearance of Circulating Blood During the Reaction, in Mice

Two methods were employed for the microscopic examination of circulating blood within the smaller blood vessels, in living mice. One group of animals was anaesthetized with nembutal, and a portion of the intact mesentery was arranged on a stage under the high-dry objective of a microscope, using the method described by Youngner and Nungester (4). In a second group, the mice were immobilized without anaesthesia upon a glass plate with scotch tape, and one ear was confined beneath the objective. In both groups, the blood vessels within a single area were observed constantly for at least one minute, and then 0.2 ml. of varying dilutions of brain tissue suspension were injected into the tail vein. Observation of the vessels was continued during the injection, and for variable periods of time thereafter.

The changes which occurred were similar in the vessels of the ear and mesentery, and were produced to an equal degree by either crude suspensions or suspensions of the sedimentable component of brain tissue.

For a period of about 20 seconds following injection, no alteration from the normal appearance of the blood within the vessels was observed. Then, at about the time when the animal, if unconfined, would be exhibiting its first symptoms, a very abrupt change occurred. The flow of blood suddenly slowed in all the vessels under observation, and ill-defined masses of apparently aggregated blood corpuscles were

seen moving sluggishly along. In some vessels the blood ceased flowing altogether, and in others it began to move back and forth in rhythmic jerking movements for a few seconds. Spasm of the vessels was not observed. The appearance of the blood at this stage resembled somewhat the so-called "sludge" phenomenon described by Knisely and his co-workers (5), in the vessels of animals under conditions of infection or trauma. Further mention of this resemblance will be made in a later section of this paper.

For about ten or fifteen seconds the blood remained in this state, either moving very slowly or not at all. After a few seconds more, the flow began again in all of the vessels under observation, at first in rhythmic jerks and then in a more even flow. Within several more seconds, the vessels and their contents appeared to be entirely normal.

When higher dilutions of the brain tissue suspension were injected, the interval preceding the change in the appearance of the blood was prolonged, and the duration of the change was briefer, but the appearance was, in general, the same as described above.

When more concentrated suspensions were injected, the flow of blood stopped completely in all vessels and death occurred in some animals within a few seconds. In two mice, which survived following the injection of a concentrated suspension, a definite thrombus was seen within one of the larger vessels, apparently a vein, after the flow of blood in other vessels had been re-established. In each instance, the thrombus appeared to be attached by a narrow base to one side of the vessel, and extended along the vessel for a short distance, filling part of the lumen. The flow of blood around the thrombus appeared to be unimpeded, and during a period of several minutes no change in the size or extent of the thrombus was observed.

Since it was shown in earlier experiments that heparin protected mice against the symptoms of ataxia and convulsions following injections of tissue suspension, it was of interest to determine whether this drug was capable of influencing the intravascular "sludging" and stasis described above. Five mice were prepared for direct visualization of mesenteric vessels, and were then injected with heparin in an amount sufficient to prevent symptoms, followed by a fully toxic dose of brain tissue suspension. No change in the normal appearance of circulating blood occurred in these animals.

Specimens of fixed tissues were obtained from mice at varying intervals after the injection of tissue suspensions, and examined for the presence of thrombi. In a few animals which received small doses of suspension and underwent a brief period of ataxia and convulsions, occasional small thrombi were encountered in the capillaries of the brain. In other animals similarly treated, no thrombi could be found in any of the tissues examined, including brain, kidney, heart, lung, liver and spleen. In animals which were injected with concentrated doses of suspension and died within a few minutes after injection, numerous thrombi were seen in the small vessels of many tissues.

Sedimentability of the Active Tissue Component

The component in crude suspensions of mouse brain tissue which produced the reaction under study was composed of particles which could be sedimented by centrifugation at high speed.

This finding is illustrated by the following experiment. A 10 per cent suspension of mouse brain tissue was centrifuged at 2,000 R.P.M. in a horizontal centrifuge for 10 minutes. The supernatant fluid was then divided into two portions. One portion was centrifuged at 12,000 R.P.M. for 30 minutes. The supernatant fluid was removed, and the packed sediment was resuspended in physiological saline to the original volume and recentrifuged at low speed to remove gross particles. Serial two-fold dilutions in saline were made with the original crude suspension, the supernatant fluid following high-speed centrifugation, and the resuspended sediment from the latter. Each of these dilutions was tested by intravenous injection in mice. The results are shown in Table IV.

It will be seen that the crude suspension produced typical reactions in a dilution of 1–160. The supernatant fluid after centrifugation of this suspension at 12,000 R.P.M. failed to produce reactions in any dilution. The reconstituted sediment possessed all of the activity which was demonstrable in the original suspension, and produced, like the latter, reactions in the dilution of 1–160.

The foregoing observations do not provide any information as to the relative size of the tissue particles which were contained in the suspensions of the sedimentable tissue component. It is obvious that centrifugation of crude tissue suspensions at 12,000 R.P.M. must bring down

particles of many different sizes. The sedimentability of the active tissue component at various speeds below 12,000 R.P.M. was found to vary in crude suspensions of different tissues. In some preparations a considerable proportion of the component could be sedimented at 8,000 R.P.M., and in a few suspensions even 5,000 R.P.M. brought down much of the component.

Occurrence of the Active Component in Other Tissues

Suspensions of the sedimentable component in a variety of other tissues, from mice, rabbits and human beings, were prepared by centrifugation of crude suspensions at 12,000 R.P.M. and resuspension of the

TABLE IV

Sedimentability of the Tissue Component Causing Ataxia and Convulsions in Mice, in a Suspension of Mouse Brain Tissue

COMPONENT OF TISSUE SUSPENSION	DILUTION OF SUSPENSION					
	1–10	1–20	1–40	1–80	1–160	1–320
Supernate: 2,000 R.P.M.	+*	+	+	+	+	0
Supernate: 12,000 R.P.M.	0	0	0	0	0	0
Sediment: 12,000 R.P.M.	+	+	+	+	+	0

* Symbols refer to the presence or absence of reactions in mice injected with the indicated dilution of tissue suspension.

sediment in saline. Serial dilutions of each suspension were then tested for the production of ataxia and convulsions in mice. The results are shown in Table V. It will be seen that the reaction was produced not only by brain but also by mouse lung, kidney, and spleen tissue. Moreover, identical reactions were produced by suspensions of the sedimentable component of rabbit brain, lung, kidney and liver tissue, and by human brain, lung, heart, kidney and spleen.

Some variation was encountered in the titers obtained with the different preparations. For example, brain and lung tissue appeared to possess more of the active tissue component than the other organs tested in each species. In contrast, the reaction was produced only by the lowest dilution of rabbit liver, and did not occur with mouse liver. The component was not demonstrable in rabbit striated muscle.

The Effect of Heat on the Activity of Tissue Suspensions

The effect of exposure to various temperatures on the activity of tissue suspensions from various sources was tested. Ten per cent suspensions of mouse brain, rabbit brain, human brain and human lung tissue were heated for 30 minutes at 56°C., 60°C., and 80°C. In each instance, both crude suspensions and suspensions of the sedimentable tissue component were tested simultaneously. After

TABLE V

Production of Ataxia and Convulsions in Mice by Suspensions of the Sedimentable Tissue Component of Various Species and Organs

TISSUE SUSPENSION		DILUTION OF TISSUE SUSPENSION					
Species	Organ	1-10	1-20	1-40	1-80	1-160	1-320
Mouse	Brain	+	+	+	+	+	0
	Lung	+	+	+	+	+	0
	Kidney	+	+	+	0	0	0
	Spleen	+	+	+	+	0	0
	Liver	0	0	0	0	0	0
Rabbit	Brain	+	+	+	+	+	0
	Lung	+	+	+	+	+	0
	Kidney	+	+	+	0	0	0
	Liver	+	0	0	0	0	0
	Striated muscle	0	0	0	0	0	0
Human	Brain	+	+	+	+	+	0
	Lung	+	+	+	+	+	0
	Heart	+	+	+	0	0	0
	Kidney	+	+	0	0	0	0
	Spleen	+	+	+	0	0	0

heating, two-fold serial dilutions of each suspension were made in physiological saline, and the titer determined by injection into mice.

The results, which differed considerably in different types of tissue suspension, are shown in Table VI. It will be seen that the crude suspension of mouse brain, which had an endpoint in the 1–80 dilution before heating, was not affected by 56°C., but was completely inactivated by heating at 60° and 80°C. In contrast, a suspension of the sedimentable component of this tissue was only slightly reduced in activity by exposure to 80°C.

TABLE VI

The Effect of Heating Various Tissue Suspensions on Their Toxicity for Mice

TISSUE	SUSPENSION	TEMPERATURE‡	DILUTION OF SUSPENSION					
			1–10	1–20	1–40	1–80	1–160	1–320
		°C.						
Mouse Brain	Crude*	Unheated	+	+	+	+	0	
		56	+	+	+	+	0	
		60	0	0	0	0		
		80	0	0				
	S.T.C.†	Unheated	+	+	+	+	0	
		56	+	+	+	+	0	
		60	+	+	+	0	0	
		80	+	+	+	0	0	
Rabbit brain	Crude	Unheated	+	+	+	+	0	
		56	+	+	+	+	0	
		60	0	0	0	0		
		80	0	0				
	S.T.C.	Unheated	+	+	+	+	0	
		56	+	+	+	+	0	
		60	+	0	0			
		80	+	0	0			
Human brain	Crude	Unheated	+	+	+	+	+	0
		56	+	+	+	+	+	0
		60	+	+	+	0	0	
		80	+	+	+	0	0	
	S.T.C.	Unheated	+	+	+	+	+	0
		56	+	+	+	+	+	0
		60	+	+	+	+	+	0
		80	+	+	+	+	+	0
Human lung	Crude	Unheated	+	+	+	+	0	
		56	+	+	+	+	0	
		60	+	+	+	+	0	
		80	+	+	+	+	0	
	S.T.C.	Unheated	+	+	+	+	0	
		56	+	+	+	+	0	
		60	+	+	+	+	0	
		80	+	+	+	+	0	

* Crude = Supernatant fluid after centrifugation at 2,000 R.P.M.

† S.T.C. = Sedimentable tissue component, after 12,000 R.P.M., resuspended in NaCl.

‡ Tissue suspensions heated for 30 minutes at the temperature indicated.

Similar results were obtained with the rabbit brain tissue suspensions. The crude suspension was not affected by 56°C., but was inactivated at higher temperatures. The suspension of the sedimentable component was partially inactivated at 60° and 80°C., but still retained activity in the 1–10 dilution.

Human brain tissue suspensions showed greater stability to heat than mouse or rabbit brain. The crude suspension, which had a titer of 1–160 before heating, was reduced in activity by 60° and 80°C., but still caused reactions in the dilution of 1–40. On the other hand, the suspension of the sedimentable component was not affected by heating at these temperatures.

TABLE VII

The Effect of Preliminary Injections of Small Amounts of Tissue Suspension upon the Susceptibility of Mice to Subsequent Injection of Toxic Doses

PRELIMINARY INJECTION	SECOND INJECTION	ATAXIA AND CONVULSIONS	
		Prepared group*	Controls
Mouse brain—dil. 1–160	Mouse brain—dil. 1–40	1/12†	12/12
Mouse brain—dil. 1–160 followed by 1–40	Mouse brain—dil. 1–10	0/11	12/12
Mouse kidney—dil. 1–80	Mouse brain—dil. 1–20	0/8	8/8
Rabbit brain—dil. 1–160	Mouse brain—dil. 1–40	0/8	8/8
Human lung—dil. 1–320	Mouse brain—dil. 1–40	1/8	8/8
Mouse brain—dil. 1–160	Human lung—dil. 1–40	0/8	8/8

* Mice which received preliminary injections of indicated tissue suspension.

† Numerator refers to number of mice showing ataxia and convulsions following the second injection. Denominator refers to number of mice tested.

Human lung tissue differed from the three varieties of brain tissue, in that both the crude and sedimentable component suspensions were completely resistant to heating at 80°C.

Induced Resistance to the Toxic Effect of Tissue Suspensions in Mice

The smallest amount of the sedimentable component of mouse brain tissue which produced ataxia and convulsions in mice was, in most preparations, represented by dilutions of 1–80 or 1–160. It was observed that when mice were injected with dilutions which were less than the amount required to produce symptoms, they rapidly became resistant to the toxic action of more concentrated suspensions.

An illustration of this phenomenon is presented in Table VII. Twelve mice were injected with a 1–160 dilution of mouse brain tissue,

with no reaction. Five minutes later, each mouse was reinjected with the same material in a dilution of 1–40, and at the same time 12 control mice were given a similar dose. Only one of the 12 animals which had received the preliminary dilution of 1–160 showed symptoms following the second injection, while all of the control mice exhibited typical ataxia and convulsions.

The 11 mice which survived the second injection without effect were reinjected five minutes later with a dilution of 1–10, and at the same time 12 control mice were similarly injected. None of the previously injected mice showed the usual reaction of ataxia and convulsions, although some of the animals appeared somewhat weak and ruffled. In contrast, all of the control animals exhibited typical, severe reactions which in several instances were rapidly fatal.

A similar state of resistance was produced when preparatory injections of tissue from one organ were followed by toxic doses of tissue suspension derived from another organ. For example, as is shown in Table VII, mouse kidney tissue in less than toxic doses provided 8 mice with resistance to the toxic action of mouse brain tissue suspension. Moreover, it will be seen that the tissues of different species produced protection against each other, as, for example, rabbit brain against mouse brain, human lung against mouse brain, and mouse brain against human lung.

The state of resistance was not produced if the preliminary injections of tissue suspension were made by any route other than intravenous. The injection of 1.0 ml. intraperitoneally and 1.0 ml. subcutaneously, of concentrated brain tissue suspension, did not cause any increase in the resistance of mice to subsequent intravenous injections of the same tissue.

Resistance did not occur if suspensions of brain tissue which had been inactivated by heating were used for the preliminary injection. It was necessary to employ active suspensions which were only slightly less concentrated than the dose required to produce obvious symptoms. For example, when a given suspension of brain tissue possessed a titer of 1–80, resistance could be induced by injecting the 1–160 dilution and sometimes by the 1–320 dilution, but no effect was demonstrable following the 1–640 dilution.

The state of resistance was first demonstrable within two or three minutes after the preliminary injection, and was present with greatest

regularity at a period between five and ten minutes following injection. After 30 minutes it was demonstrable in a smaller proportion of animals, and at the end of one hour it could no longer be demonstrated.

During the period of resistance, the injection of concentrated tissue suspensions produced little or no change in the appearance of the circulating blood in the vessels of the ear and mesentery, in contrast to the stasis and "sludging" which was observed in unprepared mice.

TABLE VIII

Effect of the Intravenous Injection of Mouse Brain Tissue Suspension on Clotting Time in Mice

DILUTION OF SUSPENSION	TIME AFTER INJECTION	CLOTTING TIME (INDIVIDUAL MICE)					
1–20	15 seconds	< 5 sec.	< 5 sec.	< 5 sec.	< 5 sec.	< 5 sec.	< 5 sec.
	5 minutes	> 2 hrs.	> 2 hrs.	> 2 hrs.	> 2 hrs.	> 2 hrs.	> 2 hrs.
	15 minutes	11 min.	8 min.	5 min.	14 min.	12 min.	10 min.
1–160	15 seconds	10 sec.	20 sec.	24 sec.	30 sec.	40 sec.	30 sec.
	5 minutes	> 2 hrs.	> 2 hrs.	> 2 hrs.	10 min.	12 min.	8 min.
	15 minutes	11 min.	8 min.	5 min.	3 min.	5 min.	3 min.
Normal mice.....		4 min.	5 min.	3 min.	4 min.	3 min.	3 min.

The Effect of Injections of Brain Tissue Suspension on the Coagulability of the Blood in Mice

In order to obtain an explanation for the phenomenon of induced resistance described in the preceding section, a study of the coagulation time of the blood of mice following injection of tissue suspensions was undertaken.

Groups of mice were injected intravenously with various dilutions of a suspension of the sedimentable component of mouse brain tissue. Clotting time determinations were made in sample mice at intervals of 15 seconds, 5 minutes, and 15 minutes after the injection, by placing 0.5 ml. of heart's blood in a small glass test tube, and tilting the tube at intervals until complete clotting was apparent. By this method the clotting time in a group of twelve normal mice was found to range between 3 and 8 minutes.

The results which were obtained in two illustrative experiments are shown in Table VIII. In the first experiment, 18 mice were injected

with 0.2 ml. of a 1–20 dilution of tissue suspension. Each of these animals developed a severe convulsive reaction, which started between 15 and 20 seconds after the injection. At this time, the clotting time in six animals was so rapid that the blood began to clot within the syringe while being drawn. The clotting time was assumed to be less than 5 seconds. Five minutes later, the clotting time in six other mice was determined. In each instance, the blood failed to clot within a period of 2 hours. Fifteen minutes after the injection, the clotting time of the remaining six mice ranged between 5 and 14 minutes.

Similar effects were produced by a smaller dose of the tissue suspension. Eighteen mice were injected with the 1–160 dilution, which caused no symptoms. Fifteen seconds later, the clotting time in six mice was much accelerated, varying between 10 and 40 seconds. At five minutes, three of the animals had clotting times longer than 2 hours, while three others were moderately prolonged. At fifteen minutes, the clotting time in the remaining six mice was normal or nearly normal.

Additional experiments of the same nature were carried out in other groups of mice with the same general results, i.e., a sharp reduction in clotting time within the first 30 seconds, followed by a marked prolongation at five minutes, and approaching normal again after fifteen minutes. These findings offer a possible explanation for the state of resistance to toxic doses of tissue suspension which followed the injection of smaller doses. It is of interest that the maximum degree of incoagulability was observed at five minutes following injection, which was also the time at which resistance to a second injection was most regularly demonstrable.

No inhibitor of coagulation could be detected in the whole blood or serum of mice at the time when the clotting time was most prolonged. When blood from these animals was mixed with fresh blood from normal mice, the clotting time of the mixture was not significantly different from that of normal blood.

Prothrombin time determinations by the method of Quick (6) were undertaken with plasma from six mice which had been injected five minutes earlier with a 1–160 dilution of tissue suspension, and compared with the prothrombin time in six normal mice. The normal prothrombin time ranged between 9 and 12 seconds. In three of the

injected mice, the prothrombin time lay within this range, while in the other three the time was 15, 16 and 17 seconds respectively. The latter figures, although somewhat prolonged, did not seem to be sufficiently abnormal to account for the marked delay in coagulation of whole blood observed in injected mice.

The Effect of Injections of Rabbit Brain Tissue Suspension on the Coagulability of the Blood in Rabbits

Normal rabbits, weighing approximately 6 pounds, were injected intravenously with various dilutions of suspensions of the sedimentable component of rabbit brain tissue, in doses of 5 or 10 ml. per rabbit. Considerable variation was encountered in the susceptibility of individual rabbits to the toxic action of tissue suspensions, as compared with the uniform responses which had been observed in mice. Further variation could be produced by varying the speed with which the suspensions were injected; rapidly introduced suspensions caused symptoms more frequently than slow injections. When symptoms occurred, they consisted of severe generalized convulsions which usually began within 30 and 60 seconds after the injection and lasted intermittently for about 1 minute. In almost every instance, the convulsions were followed by immediate death, and were associated with widespread intravascular clotting.

Blood was obtained from the heart several minutes before the injection of tissue suspension, and at varying intervals following injection. Clotting time was determined with 0.5 ml. samples, using the method previously described. The clotting time in twenty-five normal rabbits ranged between 3 and 10 minutes.

The results obtained in a series of 25 rabbits were not uniform, due largely to the difficulty of selecting an adequate dose of tissue suspension for individual animals without causing generalized convulsions and death. However, in 12 rabbits of this series, definite changes in the coagulability of blood were observed following injection, and these changes were similar to those observed in mice. Within the first minute after injection the clotting time became accelerated, and five minutes later it was prolonged beyond normal. The delay in coagulation persisted for varying lengths of time during the following hour.

A detailed summary of the changes in clotting time in four rabbits is shown in Table IX. Each of these animals was injected with 10 ml. of a 1–40 dilution of rabbit brain sedimentable tissue suspension. It will be seen that the first animal had a clotting time of 8 minutes

TABLE IX

Effect of the Intravenous Injection of Rabbit Brain Tissue Suspension on Clotting Time and Prothrombin Time in Rabbits

RABBIT NO.	DILUTION OF SUSPENSION	BLOOD OBTAINED	CLOTTING TIME	PROTHROMBIN TIME
				sec.
1	1–40	Before injection	8 minutes	15
		After 30 seconds	3 minutes	16
		After 5 minutes	21 minutes	14
		After 15 minutes	22 minutes	14
		After 30 minutes	> 2 hours	29
		After 45 minutes	20 minutes	13
		After 60 minutes	14 minutes	13
2	1–40	Before injection	6 minutes	12
		After 30 seconds	1 minute	
		After 10 minutes	48 minutes	12
		After 30 minutes	> 2 hours	60
		After 50 minutes	4 minutes	12
3	1–40	Before injection	6 minutes	14
		After 30 seconds	30 seconds	
		After 5 minutes	23 minutes	12
		After 15 minutes	27 minutes	14
		After 30 minutes	7 minutes	14
4	1–40	Before injection	5 minutes	
		After 1 minute	1¾ minutes	
		After 5 minutes	> 2 hours	
		After 20 minutes	> 2 hours	
		After 60 minutes	4 minutes	

before injection. Thirty seconds after injection the time was 3 minutes. At five and fifteen minutes after injection, the clotting time had increased to approximately 20 minutes, and at a half-hour the blood failed to clot within 2 hours. At forty-five minutes the clotting time had decreased to 20 minutes, and at the end of the hour it was close to normal. Comparable results were obtained in the other three

rabbits indicated in Table IX, although some variation was encountered in the time intervals at which maximum prolongations of clotting time occurred.

The prothrombin times in successive plasma samples from three of the rabbits are shown in Table IX. It will be seen that the prothrombin time remained within normal limits in all except two samples. In rabbit no. 1 the prothrombin time one-half hour after injection was 29 seconds, and in rabbit no. 2, at the same interval, the time was 60 seconds. In each instance, the plasma clots were loose and granular, as compared with the firm clots observed in other plasma specimens.

No anticoagulant substance could be demonstrated in the blood of rabbits following the injection of tissue suspensions. When the relatively incoagulable blood of these animals was mixed with normal rabbit blood, no delay in the clotting time of the latter was observed. Moreover, the clotting time was not shortened by the addition of varying concentrations of salmine, which is known to act as an antagonist of heparin (7). Platelet counts remained normal throughout the reaction in three rabbits tested.

DISCUSSION

It has long been recognized that the intravenous injection of tissue extracts in animals may result in the formation of extensive thromboses throughout the vascular compartment, causing death within a few minutes. Such an event can be produced in mice, if considerable doses of a concentrated tissue suspension are injected. Smaller doses of the same material, however, bring about a characteristic, almost stereotyped pattern of neurological symptoms, which are transient and apparently completely reversible. Between the time of injection and the appearance of symptoms, a latent interval is always observed. The duration of this interval is quite regular and constant, for a given dose of tissue suspension, and varies directly with the concentration of material injected.

At the time when symptoms appear in the mice, certain changes occur in the physical state of the circulating blood, and can be observed directly by microscopic examination of the vessels of the ear or mesentery. The blood slows, becomes "sludge-like" in appearance, and may come to a complete standstill in many vessels. Then, after a period of seconds, it moves on again in a normal fashion.

This reaction does not occur if the mice are treated beforehand with heparin, or with anticoagulant doses of congo red. It does not occur if the tissue suspension has been inactivated by heating. Moreover, if the mice have received a preliminary sub-toxic injection of the tissue suspension they become, within a few minutes, resistant to fully toxic amounts; this state of resistance is associated with a marked prolongation of the coagulation time.

The tissue component which is responsible for the reaction in mice is sedimentable by centrifugation at high speed, and is demonstrable in a wide variety of homologous and heterologous tissues. Similar tissue components are known to contain thromboplastin (8). It is reasonable to assume that the symptoms, and the transient stasis of blood flow, are both caused by the thromboplastic action of the tissue suspensions introduced into the circulating blood, and that the acquired resistance displayed by previously injected animals is the result of the temporary coagulation defect which occurs following such injections.

The predominantly neurological character of the symptoms in mice suggests an injury to the brain. The physiological basis of this injury is, however, not clear. Is it due to actual thrombosis within cerebral vessels? If so, it is somewhat difficult to understand the rapidity and completeness with which many of the mice recover, following the reaction. Also, one might expect to find more histological evidence of thrombosis than was encountered in the brain sections examined. These objections, however, are not sufficient to exclude the possibility of cerebral thrombi as the underlying basis for the reaction. It is conceivable that scattered, small thrombi, if accompanied by temporary circulatory stasis elsewhere in the body, or if attended by local vasospasm within the brain, might account for the transient symptoms observed. Moreover, such thrombi might be extremely difficult to locate in fixed brain sections.

However, it is also possible that the same sort of intravascular changes which were observed by direct visualization, in the ear and mesenteric vessels, may take place within the brain. There may be stasis and "sludging" of blood, without necessarily the formation of organic, fixed thrombi, and such a change may result in a severe enough degree of cerebral ischemia to account for the symptoms displayed by the animals. On such a basis, it would be easier to explain

the transient and reversible course of the reaction as well as the failure to detect thrombi in fixed tissue sections. The nature of this disturbance in blood flow is, at the present time, a matter for speculation. Whether it is due to an increase in viscosity, or to obstructive aggregations of the particulate elements of the blood, or to an actual change in the colloidal state of the blood representing "partial clotting", has not been determined.

The theoretical implications of such an explanation are considerable, and perhaps a certain amount of correlary speculation is warranted. It is conceivable that a similar event may take place under other circumstances, during the course of disease or as the result of trauma, and may be the basis for certain types of tissue damage. Injury to the endothelial barrier between the blood and tissues, for example, could result in the release of a similar component of tissue into the local vascular bed, producing a local, transient disturbance in blood fluidity with the temporary effect of thrombosis, but without the formation of an actual thrombus. Moreover, it is known that normal blood contains thromboplastic substances, which are demonstrable in platelets and perhaps also in erythrocytes and white cells (9). Liberation or activation of these sources of thromboplastin might also lead to similar disturbances in blood flow.

It has already been mentioned that the blood flow changes in mice seemed to resemble the "sludging" described by Knisely and his associates (5) in the small blood vessels of animals in the course of certain acute infections, notably malaria in monkeys, and also in vessels adjacent to traumatized areas in mice. The possibility of a similar underlying mechanism seems of sufficient interest to warrant mention at this time.

In this connection it is also of interest to consider the recent studies of Friedman, Lange and Weiner on experimental frostbite and gangrene in animals (10). These workers found that the freezing of living tissue was followed by changes in the physical state of blood within the vessels of the affected area, which resembled "sludging". Gangrene of the area was prevented by the administration of heparin. It is possible that the trauma of freezing may have caused the release of thromboplastic material into the local vascular bed, resulting in blood flow changes analogous to those observed in mice following the injection of tissue suspensions.

The occurrence of decreased coagulability in animals following the injection of tissue extracts has been recognized for many years. In 1886, Wooldridge (1) reported that the injection of "tissue fibrinogen" intravenously in dogs resulted in intravascular clotting when large quantities were given. The injection of smaller doses was followed by a period in which the blood was relatively incoagulable, and during which the animals were insusceptible to the toxic effect of larger doses of the tissue extract. In 1909, similar observations were made by Mellanby (2), employing thromboplastic snake venoms instead of tissue extracts. This worker explained the increase in coagulation time on the basis of a depletion in circulating fibrinogen, resulting from intravascular clotting initiated by the injected material. In the present study, employing mice and rabbits, no new explanation of the coagulation changes has been advanced. However, it would appear that the explanation offered by Mellanby cannot be applied to the results obtained here, since the prolongation of clotting time was, in most instances, associated with a normal prothromin time and normal clot formation. In two exceptions noted, an increase in prothrombin time appeared 30 minutes after the injection of tissue suspension in rabbits, but in both instances the prothrombin time in earlier plasma specimens was normal.

Prolongations of the coagulation time are also known to occur following anaphylactic shock in animals, and after the shock which results from injections of peptone. There is evidence that in both instances the incoagulability is due to the liberation of anticoagulant substances (11, 12). The physiological events which precipitate the characteristic symptoms in these two varieties of shock are still obscure. It is of interest that some workers have reported the inhibition of anaphylactic shock by heparin (13).

The reproducible and easily recognized symptoms displayed by mice following the injection of tissue suspensions made it possible to titrate the *in vivo* thromboplastic activity of the suspensions in a more or less quantitative fashion, thus providing a method for studying some of the properties of the tissue component involved. The observed changes in thermostability following high-speed centrifugation of crude brain tissue suspensions are of interest. Crude suspensions of mouse brain tissue were inactivated by heating at 60°C., while saline suspensions of the component sedimented by 12,000 R.P.M.

were resistant to the effect of 80°C. This observation is unexplained at the present time, but it suggests that the active component may be linked, in crude suspensions, to a thermolabile factor from which it is separated by centrifugation. It has been reported by Kidd and Friedewald (14) that a similar increase in thermostability following centrifugation occurred in an antigenic component of normal tissue which reacted in complement fixation tests with normal rabbit serum. The possible relationship between this antigenic component and the thromboplastic tissue component under study will be discussed in the paper which follows (15).

SUMMARY

The intravenous injection of saline suspensions of normal mouse brain tissue, in mice, caused a characteristic and reproducible reaction consisting of ataxia, convulsions and coma. The reaction was accompanied by transitory changes in the appearance of the circulating blood in the vessels of the ear and mesentery, with slowing and, in some vessels, complete cessation of blood flow. These changes were usually followed, after a short interval, by the resumption of normal blood flow.

The reaction in mice was prevented by the administration of heparin, and also by congo red in anticoagulant doses.

The tissue component which caused the reaction was completely sedimentable by centrifugation at 12,000 R.P.M., and was demonstrable in a variety of mammalian tissues. Crude suspensions of brain tissue were inactivated by heating at 60°C. Suspensions of the brain tissue component after centrifugation at high speed were resistant to temperatures as high as 80°C.

When mice were injected with small amounts of the tissue component they became, within a few minutes, resistant to the effect of fully toxic doses of the same material. This state of resistance persisted for less than one hour.

Following the injection of tissue suspensions in mice and rabbits, the clotting time of the blood was at first accelerated, then became markedly prolonged, and then returned to normal within one hour after injection.

Some of the theoretical implications of these observations are discussed.

BIBLIOGRAPHY

1. Wooldridge, L. C.: Arch. f. Anat. u. Physiol. (Phys. Abt.), 1886, p. 397.
2. Mellanby, J.: J. Phys., 1909, **38,** 442.
3. Talliaferro, I., and Haag, H. B.: Am. J. Med. Sci., 1937, **193,** 626.
4. Youngner, J. S., and Nungester, W. J.: J. Inf. Dis., 1944, **74,** 247.
5. Knisely, M. H., Eliot, T. S., and Bloch, E. H.: Arch. Surg., 1945, **51,** 220.
6. Quick, A.: The Hermorrhagic Diseases and the Physiology of Hemostasis. C. C. Thomas, Springfield, Ill., 1942.
7. Chargaff, E., and Olson, K. B.: J. Biol. Chem., 1937, **122,** 153.
8. Chargaff, E.: Advances in Enzymology, Vol. V. Interscience Publishers, Inc., New York, N. Y., 1945.
9. Silberberg, M.: Phys. Rev., 1938, **18,** 197.
10. Friedman, N. B., Lange, K., and Weiner, W.: Am. J. Med. Sci., 1947, **213,** 61.
11. Eagle, H., Johnston, C. C., and Ravdin, J. S.: Bull. Johns Hopkins Hospital, 1937, **60,** 428.
12. Jacques, L. B., and Waters, E. T.: J. Physiol., 1941, **99,** 454.
13. Macht, D. I.: Ann. Int. Med., 1943, **18,** 772.
14. Kidd, J. G., and Friedewald, W. F.: J. Exp. Med., 1942, **76,** 543.
15. Thomas, L.: Bull. Johns Hopkins Hospital, 1947, **81,** 26.

STUDIES ON THE INTRAVASCULAR THROMBOPLASTIC EFFECT OF TISSUE SUSPENSIONS IN MICE

II. A Factor in Normal Rabbit Serum Which Inhibits the Thromboplastic Effect of the Sedimentable Tissue Component

LEWIS THOMAS.

From the Department of Pediatrics, Johns Hopkins University Medical School and The Harriet Lane Home for Invalid Children, Baltimore, Maryland

In a previous paper (1) evidence was presented which indicates that the characteristic reaction of ataxia, convulsions and coma, which is produced in mice by the intravenous injection of tissue suspensions, is due to the thromboplastic action of these suspensions *in vivo*. In animals receiving lethal doses of this material, generalized intravascular clotting occurs. With smaller doses, the coagulation time is decreased at the time when the symptoms which comprise the reaction occur. The reaction may be prevented by small doses of heparin, or by anticoagulant doses of congo red. The component of tissue causing the reaction is entirely sedimentable by centrifugation at high speed.

The present paper deals with the inactivation of the sedimentable tissue component by a factor in normal rabbit serum, which is demonstrable when saline suspensions of tissue particles are incubated with serum. It will be shown that the inhibiting factor is dependent upon the presence of calcium for its effect. Certain properties of the serum factor will be described.

MATERIALS AND METHODS

Serum. Normal adult rabbits, weighing approximately six pounds each, were bled from the heart in amounts of 20 ml. each. The blood was allowed to clot at room temperature, then centrifuged at 2000 R.P.M. for 10 minutes. For prolonged storage, the serum was kept in sterile lusteroid tubes at $-70°C$. In some experiments, in which the serum was to be used within 3–4 days, it was stored in the refrigerator at 4°C.

Plasma. Rabbit plasma was obtained by adding whole blood to one-tenth of its volume of 0.1 M potassium oxalate, and centrifuging

at 2000 R.P.M. for 10 minutes. In all experiments involving plasma, fresh specimens were used on the same day as obtained.

Tissue Suspensions. Saline suspensions of the sedimentable tissue component of rabbit brain, rabbit lung, mouse brain, human lung, and human brain tissue were prepared in the following manner. Freshly obtained tissues, or specimens which had previously been stored at −70°C., were weighed, ground in a mortar with sterile alundum, and suspended in a sufficient quantity of physiological saline to produce a concentration of 10 per cent. The suspension was centrifuged at 2000 R.P.M. for 10 minutes, and then recentrifuged at 12000 R.P.M. for 30 minutes in an angle centrifuge, at 4°C. The sediment obtained by the latter centrifugation was resuspended in physiological saline to its original volume, and the resulting suspension distributed in lusteroid tubes. All suspensions were either used in experiments on the same day as prepared, or were stored in the frozen state at −70°C.

Titration of Suspensions. The potency of each suspension was determined by preparing serial two-fold dilutions in physiological saline solution, and injecting 0.2 ml. of each dilution intravenously into two mice. The highest dilution which produced the typical reaction of ataxia and convulsions was designated as one unit. In the text and tables which follow, all dilutions are referred to on the basis of the original weight of whole tissue.

Inhibition Tests. Rabbit sera were tested in the following manner for their capacity to inhibit the activity of the sedimentable tissue component. Serial two-fold dilutions of serum were prepared in physiological saline solution, or in other diluents to be described later. To each dilution of serum was added an equal volume of the tissue suspension under study. In most experiments, the concentration of the suspension was such that the final mixture of serum and tissue to be tested contained two units of the sedimentable tissue component. For example, with suspensions which produced reactions with an endpoint in the 1–160 dilution, the final concentration of tissue suspension in each mixture was 1–80.

The mixtures of serum and tissue were placed in a water-bath at 37°C. for 1 hour, after which they were tested in mice by the intravenous injection of 0.2 ml. In all tests, appropriate control tubes of serum alone and tissue alone were included. The results in each titra-

tion of serum were recorded in terms of the final dilution of serum in each tube. The reactions in mice were recorded as "plus" if the typical reaction of ataxia and convulsions occurred, and as "zero" if no reaction occurred. At least two mice were tested with each mixture, and usually three or four mice were used for the mixtures which determined the endpoint of titrations.

EXPERIMENTAL RESULTS

Inhibition of Rabbit Brain Tissue Suspensions by Rabbit Sera

Sera from 50 normal rabbits were tested for inhibition of the toxic effect of suspensions of rabbit brain tissue particles, by the method described above. An inhibiting factor was demonstrable in all of these sera, with little difference in titer between individual sera.

The inhibition of varying concentrations of tissue suspension by four samples of rabbit serum is shown in Table I. The suspension used here caused ataxia and convulsions in a dilution of 1–160, and no reactions with higher dilutions. With the 1–20 dilution, which represented eight units of tissue suspension, inhibition was produced by each serum in a dilution of 1–4. With two units of suspension the serum titer rose to 1–16, and with one unit to 1–32 in three of the sera.

The length of time required for the inhibition reaction was determined in the following manner. Serial dilutions of rabbit serum were mixed with two units of rabbit brain suspension and placed in a waterbath at 37°C. Samples of the mixture were tested in mice after one minute, and at various intervals thereafter. The results are shown in Table II. No inhibition was demonstrable one minute after preparation of the mixtures. Inhibition was demonstrable in the 1–2 dilution of serum after 5 minutes, in the 1–8 dilution after 30 minutes, and in the 1–16 dilution after 1 hour. No further increase in titer occurred after four hours of incubation.

Inhibition of Tissue Suspension from Sources Other than Rabbit Brain

Several specimens of rabbit serum were tested for their inhibitory action on tissue particles prepared from sources other than rabbit brain, including rabbit lung, mouse brain, human lung, and human brain. In each instance, two units of tissue suspension were employed. The results in all sera were essentially the same, and an illustrative experiment

is shown in Table III. It will be seen that serum produced inhibition of rabbit brain and lung, and mouse brain suspensions, in dilutions of

TABLE I

Inhibition by Normal Rabbit Sera of the Capacity of Rabbit Brain Tissue Suspensions to Produce Ataxia and Convulsions in Mice

DILUTION OF TISSUE SUSPENSION‡	RABBIT SERUM	DILUTION OF RABBIT SERUM†					
		1-2	1-4	1-8	1-16	1-32	1-64
1-20	1	0*	0	+	+	+	+
	2	0	0	+	+	+	+
	3	0	0	+	+	+	+
	4	0	0	+	+	+	+
1-80	1	0	0	0	0	+	+
	2	0	0	0	0	+	+
	3	0	0	0	0	+	+
	4	0	0	0	0	+	+
1-160	1	0	0	0	0	0	+
	2	0	0	0	0	0	+
	3	0	0	0	0	+	+
	4	0	0	0	0	0	+

* 0 = No reaction in mice following injection.
\+ = Ataxia and convulsions.
† Dilution figures refer to the final dilution of serum in each mixture.
‡ Dilution figures refer to the final dilution of the sedimentable tissue component, based on the original weight of whole tissue.

TABLE II

Effect of Time of Incubation on the Inhibition of Rabbit Brain Tissue Particles by Normal Rabbit Serum

TIME OF INCUBATION AT 37°C.	DILUTION OF SERUM				
	1-2	1-4	1-8	1-16	1-32
1 Min.	+*	+	+		
5 Min.	0	+	+		
30 Min.	0	0	0	+	+
60 Min.	0	0	0	0	+
240 Min.	0	0	0	0	+

* + = Ataxia and convulsions following injection.
0 = No reaction.

1-8 or higher. In contrast, no inhibition of human lung tissue suspension was demonstrable, and only slight inhibition of human brain.

Thermostability of the Inhibiting Factor in Rabbit Serum

The effect of heat on the property of serum to inhibit tissue particles was next investigated. Samples of serum were heated for 30 minutes at 56°, 60° and 65°C., and then tested, in two-fold dilutions, for their capacity to inhibit two units of rabbit brain suspension. The results

TABLE III

Inhibition by Rabbit Serum of Tissue Particles from Various Organs

TISSUE SUSPENSION†	DILUTION OF RABBIT SERUM					
	1-2	1-4	1-8	1-16	1-32	1-64
Rabbit Brain	0*	0	0	0	+	+
Rabbit Lung	0	0	0	+	+	+
Mouse Brain	0	0	0	0	0	+
Human Brain	0	+	+	+		
Human Lung	+	+	+	+		

* 0 = No reaction following injection.
\+ = Ataxia and convulsions.
† Two units of tissue suspension employed in each instance.

TABLE IV

Effect of Heat on the Capacity of Rabbit Serum to Inhibit Two Units of Rabbit Brain Tissue Suspension

TEMPERATURE†	SERUM DILUTION				
	1-2	1-4	1-8	1-16	1-32
Unheated	0*	0	0	0	+
56°C.	0	0	0	0	+
60°C.	0	+	+	+	+
65°C.	+	+	+	+	+

* 0 = No reaction following injection.
\+ = Ataxia and convulsions.
† Rabbit serum heated for 30 minutes at temperature indicated.

are shown in Table IV. Heating at 56°C. did not affect the inhibiting property. At 60°C., the titer was reduced from 1–16 to 1–2. At 65°C. the inhibiting property was eliminated.

The Effect of Calcium and of Oxalate on the Inhibitor in Serum

Oxalated plasma from normal rabbits was tested for the inhibition of rabbit brain tissue particles. No inhibition could be demonstrated

in plasma, as is shown in Table V, although serum prepared from the same blood samples exhibited inhibition in a titer of 1–16. Furthermore, the addition of potassium oxalate to serum, in an amount equivalent to that present in oxalated plasma, resulted in the complete disappearance of the inhibitory property of the serum within a few minutes. Equivalent amounts of potassium oxalate in physiological saline or serum produced no symptoms when injected intravenously into mice.

TABLE V

Comparison of the Capacity of Rabbit Sera and Oxalated Plasma to Inhibit Rabbit Brain Tissue Suspension

RABBIT	SPECIMEN†	DILUTION OF SERUM OR PLASMA					
		1-2	1-4	1-8	1-16	1-32	1-64
1	Serum	0*	0	0	0	+	+
	Plasma	+	+	+	+	+	+
2	Serum	0	0	0	0	+	+
	Plasma	+	+	+	+	+	+
3	Serum	0	0	0	0	+	+
	Plasma	+	+	+	+	+	+
4	Serum	0	0	0	0	+	+
	Plasma	+	+	+	+	+	+

* 0 = No reaction following injection.
\+ = Ataxia and convulsions.
† Serum and plasma obtained at same bleeding from each rabbit.

The sera which had been inactivated by oxalate could be restored to their original activity by the addition of calcium chloride. Moreover, calcium chloride alone, in small amounts, enhanced the inhibitory property of serum for tissue suspensions. These observations are illustrated in Table VI. The serum used in this experiment had an inhibiting titer of 1–16 against two units of rabbit brain suspension. The addition of 0.1 ml. of 0.1 M potassium oxalate to 0.9 ml. of this serum resulted in the disappearance of the inhibiting property. When calcium chloride was added to the mixtures of oxalated serum and tissue suspension, in an amount sufficient to produce a 0.025 molar concentra-

tion of $CaCl_2$, inhibition was demonstrable in the 1–32 dilution of serum.

Smaller amounts of calcium chloride were then tested for their effect on the inhibiting property of serum in the absence of oxalate. Varying concentrations of calcium chloride were added to mixtures of tissue particles and diluted serum before incubation, and the mixtures were tested after standing for 1 hour at 37°C. It will be seen in Table VI that the 0.00025 molar concentration of $CaCl_2$ was sufficient to cause

TABLE VI

Effect of Oxalate and Calcium on the Capacity of Rabbit Serum to Inhibit Rabbit Brain Tissue Suspension

K OXALATE IN SERUM	$CaCl_2$ IN EACH TUBE	SERUM DILUTION								
		1–2	1–4	1–8	1–16	1–32	1–64	1–128	1–256	1–512
0	0	0*	0	0	0	+	+	+	+	+
0.01 M†	0	+	+	+	+	+	+	+	+	+
0.01 M	0.025 M‡	0	0	0	0	0	+	+	+	+
0	0.025 M	0	0	0	0	0	0	+	+	+
0	0.0025 M	0	0	0	0	0	0	0	+	+
0	0.00025 M	0	0	0	0	0	0	0	+	+
0	0.000025 M	0	0	0	0	+	+	+	+	+
0	0.0000025 M	0	0	0	0	+	+	+	+	+

*0 = No reaction following injection.

\+ = Ataxia and convulsions.

† Indicates molar concentration of K oxalate in undiluted serum.

‡ Indicates molar concentration of $CaCl_2$ in each mixture of diluted serum and tissue suspension.

an increase in the titer of this serum from 1–16 to 1–128. One-tenth of this amount of $CaCl_2$ had no enhancing effect.

Calcium chloride alone, in the absence of serum, had no effect on the toxicity of tissue suspensions for mice, when tested under the same conditions and in concentrations similar to those employed in the above experiments.

These observations indicated that the presence of calcium was necessary for the action of the inhibiting factor in rabbit serum. The apparent enhancement of inhibition, which resulted from adding $CaCl_2$ to serial dilutions of serum, was interpreted as indicating that the level of effective calcium in normal serum was passed when a serum was

diluted higher than 1–16, while the level of the serum inhibiting factor extended as far as the dilution of 1–128.

The effect of removing calcium from normal serum by dialysis was next investigated. 5.0 ml. of rabbit serum was dialyzed, in a cellophane sac, against physiological saline solution for 48 hours, at 4°C. The dialyzed serum was compared with a sample of the original serum in inhibition tests against 2 units of rabbit brain suspension. The results are shown in Table VII. It will be seen that dialysis resulted in the complete loss of the inhibiting property of serum. The addition

TABLE VII

Loss of inhibitory Property of Serum after Dialysis, and Restoration of the Property by $CaCl_2$ and Heat-Inactivated Serum

SERUM	SERUM DILUTION					
	1-2	1-4	1-8	1-16	1-32	1-64
Untreated	0*	0	0	0	+	+
Dialyzed	+	+	+	+	+	+
Dialyzed + $CaCl_2$†	0	0	0	0	+	+
Dialyzed + Heated Serum‡		0	0	0	+	+
Heated Serum§	+	+	+	+	+	+

* 0 = No reaction following injection.
\+ = Ataxia and convulsions.
† 0.025 M $CaCl_2$ contained in each mixture of dialyzed serum and tissue suspension.
‡ Equal mixtures, in the dilutions indicated, of dialyzed serum and serum heated at 65°C. for 30 minutes.
§ Serum heated at 65°C. for 30 minutes.

of $CaCl_2$ to the dilutions of dialyzed serum, in a concentration of 0.025 M per tube, caused the restoration of the inhibiting property to its original titer. The inhibiting property of dialyzed serum was also restored by mixing this serum with an equal volume of rabbit serum which had been inactivated by heating at 65°C. for 30 minutes.

It was evident that the factor which was removed from serum by dialysis, and which could be replaced by the addition of calcium chloride, was also contained in serum which had been heated at 65°C. It was therefore of interest to determine whether heated serum could be employed as a diluent for the titration of the inhibitory property in fresh serum. Accordingly, serial dilutions of rabbit serum were pre-

pared in a diluent consisting of a 1–2 dilution of the same serum, which had previously been heated at 65° for 30 minutes. At the same time, comparable dilutions of the fresh serum were made in physiological saline solution. Both rows of diluted serum were then tested for the inhibition of 2 units of rabbit brain suspension. The results are shown in Table VIII. It will be seen that the serum possessed an endpoint of 1–16 when diluted in saline, and an endpoint of 1–128 when diluted in heat-inactivated serum. The latter diluent had no inhibiting effect when tested alone.

In summary, the inhibiting property in rabbit serum was not demonstrable in the presence of oxalate, or in serum which had been dialyzed

TABLE VIII

Titer of Inhibiting Factor in Rabbit Serum with Heat-Inactivated Rabbit Serum as Diluent, Compared with Titer in Saline Diluent

DILUENT	SERUM DILUTION‡						
	1–8	1–16	1–32	1–64	1–128	1–256	1–512
Heated Serum†	0*	0	0	0	0	+	+
NaCl	0	0	+	+	+	+	+

* 0 = No reaction following injection.
\+ = Ataxia and convulsions.
† Serum heated at 65°C. for 30 minutes, and used in a dilution of 1–2.
‡ Figures refer to the final dilution of fresh rabbit serum in the indicated diluent, mixed with two units of rabbit brain tissue suspension.

against physiological saline. The property was restored by the addition of calcium chloride to either the oxalated or the dialyzed serum. It was also restored when dialyzed serum was mixed with serum which had been inactivated by heating at 65°C. When calcium chloride was added to serial dilutions of serum, or when the serum dilutions were made in a diluent of heat-inactivated serum, the titer of the inhibiting property was increased from 1–16 to 1–128. These observations suggest that the inhibition of the toxicity of tissue suspensions for mice is due to the combined action of two factors in rabbit serum, one of which is inactivated by heating at 65°C., and the other a thermostable component which consists, in part if not entirely, of calcium. By themselves, neither of the components causes inhibition of tissue suspensions.

Fractionation of Rabbit Serum and Identification of Inhibiting Factor in Globulin

It was next of interest to determine whether the inhibiting factor in serum was present in the albumin or globulin fraction. Accordingly, fractionation of a sample of normal rabbit serum was carried out by precipitation with ammonium sulfate.

9.0 ml. of rabbit serum was diluted with an equal volume of physiological saline solution, and divided into two portions of 9.0 ml. each. 3.0 ml. of saturated ammonium sulfate was added to one portion, and 9.0 ml. to the other, producing, respectively, 25 per cent and 50 per cent saturation. The mixtures were kept at 37°C. for one hour, with occasional shaking, and then centrifuged at 12000 R.P.M. for 15 minutes. The globulin precipitates were each redissolved in 4.5 ml. of physiological saline. The supernatant fluid from the 50 per cent saturated serum was brought to full saturation by the addition of crystals of ammonium sulfate, then kept at 37°C. for 1 hour, and centrifuged at 12000 R.P.M. The resulting albumin precipitate was redissolved in 4.5 ml. of physiological saline. Each fraction was then dialyzed against physiological saline solution for 72 hours, at 4°C. Following dialysis, each fraction was made up to 9 ml. by the addition of saline.

Two-fold dilutions of each fraction were tested for the inhibition of two units of rabbit brain suspension. No inhibitory action was demonstrable in any fraction when physiological saline was used as a diluent. However, since it had been shown that dialysis of whole serum caused the inhibiting property to disappear, and the property could be restored by adding heat-inactivated serum, a 1–2 dilution of serum which had been heated at 65°C. was employed as diluent instead of saline. The results of inhibition tests with this diluent are shown in Table IX. It will be seen that the inhibiting factor was confined to the globulin fraction which was precipitated at 50 per cent saturation with ammonium sulfate. No inhibiting property was demonstrable in the fraction of globulin which was precipitated at 25 per cent saturation, or in the albumin fraction.

Removal of the Inhibiting Property by Absorption with Tissue Particles

It was found that the inhibiting property could be partially removed from rabbit serum by absorption with rabbit brain tissue particles. This observation is illustrated by the following experiment.

20.0 ml. of a 10 per cent crude suspension of rabbit brain tissue were centrifuged at 12000 R.P.M. for 30 minutes. The sediment was suspended in 1 ml. of normal rabbit serum. This mixture was placed at 37°C. for two hours, followed by 18 hours at 4°C., after which it was

TABLE IX

Distribution of the Inhibiting Factor in Rabbit Serum

FRACTION OF SERUM†	DILUTION OF FRACTION‡					
	1-4	1-8	1-16	1-32	1-64	1-128
Globulin (25 per cent saturation).....	+*	+	+	+	+	+
Globulin (50 per cent saturation).....	0	0	0	0	+	+
Albumin.....	+	+	+	+	+	+
Whole Serum.....	0	0	0	0	0	+

* + = Ataxia and convulsions following injection.
0 = No reaction.

† Obtained by precipitation with ammonium sulfate.

‡ All dilutions of serum fractions were made in a diluent of rabbit serum which had been heated at 65°C. for 30 minutes.

TABLE X

Absorption of Rabbit Serum Inhibitor by Rabbit Brain Suspension

SERUM	TISSUE SUSPENSION TESTED	DILUTION OF SERUM				
		1-4	1-8	1-16	1-32	1-64
Absorbed with Rabbit Brain Tissue	Rabbit Brain	0*	+	+	+	+
	Mouse Brain	0	+	+	+	+
Unabsorbed Control	Rabbit Brain	0	0	0	0	+
	Mouse Brain	0	0	0	0	+

* 0 = No reaction following injection.
+ = Ataxia and convulsions.

centrifuged at 12000 R.P.M. for 30 minutes. The supernatant serum was tested for its capacity to inhibit 2 units of rabbit brain suspension, and compared with a sample of untreated serum which had been subjected to the same temperatures and centrifugation. In addition, both sera were tested for the inhibition of 2 units of mouse brain suspension. The results are shown in Table X. It will be seen that the unabsorbed serum possessed an inhibitory titer of 1–16 for both suspensions of brain

tissue. Following absorption with rabbit brain suspension, the end-point was reduced to 1–4, against both suspensions.

Indirect evidence suggesting that an inhibiting factor may have been removed from serum by the tissue particles was obtained in the following manner. The absorption experiment was repeated, using the method described above. After absorption, the tissue particles which were separated from the serum by centrifugation were resuspended in 20 ml. of physiological saline, thus restoring the original 10 per cent concentration. Two-fold dilutions of the suspension were made in saline, and the titer determined by injecting 0.2 ml. of each dilution into mice. At the same time, for comparison, the titer of the original suspension, which had not been exposed to rabbit serum, was determined.

TABLE XI

Inactivation of Rabbit Brain Tissue Particles by Serum, and Restoration of Activity of Particles by Oxalate

TISSUE SUSPENSION	DILUTION OF TISSUE SUSPENSION				
	1-10	1-20	1-40	1-80	1-160
Untreated	+*	+	+	+	0
Serum-treated†	0	0	0	0	0
Serum-treated† + Oxalate‡	+	+	+	0	0

* + = Ataxia and convulsions following injection.
0 = No reaction.
† Tissue particles which had previously been incubated with rabbit serum (see text).
‡ Potassium Oxalate 0.01 M.

It was found that the toxicity of the tissue particles used for absorption with rabbit serum had disappeared, even in the 1–10 dilution. In contrast, the original suspension produced ataxia and convulsions in a dilution of 1–80. Potassium oxalate, in a 0.1 M solution, was now added, in the proportion of 1–10, to the tissue suspension which had apparently been inactivated. After several minutes, the titer of this suspension was again determined by the injection of serial dilutions into mice. The results are shown in Table XI. The addition of oxalate caused the reappearance of toxicity, now demonstrable in a dilution of 1–40.

These results indicated that an inhibiting factor in serum was still associated with the tissue particles after removal of the latter from serum, and that the inhibition was reversible by potassium oxalate.

Attempts to Demonstrate Inhibition of Brain Tissue Suspension in vitro

Evidence has been presented in a previous paper which indicates that the reaction of mice to the injection of tissue suspensions is due to the thromboplastic effect of this material *in vivo*. It is known that tissue particles such as those employed in the present experiments contain thromboplastin, the activity of which can be demonstrated in recalcified plasma. It seemed of importance to determine whether

TABLE XII

Comparison of the In Vitro *Thromboplastic Action and the Production of Reactions in Mice, by Mixtures of Rabbit Brain Tissue Suspensions and Rabbit Serum*

DILUTION OF TISSUE SUSPENSION	TEST	SERUM DILUTIONS							NaCl CONTROL†
		1-2	1-4	1-8	1-16	1-32	1-64	1-128	
1-20	Clotting Time	16″*	15″	15″	14″	13″	15″	15″	17″
	Mouse Injection	0‡	0	0	+	+	+	+	+
1-160	Clotting Time	25″	26″	28″	21″	18″	18″	18″	22″
	Mouse Injection	0	0	0	0	0	+	+	+

* Figures refer to clotting time of recalcified rabbit plasma, expressed in seconds, produced by the mixtures of tissue suspension and the indicated serum dilutions.

† Tissue suspension mixed with physiological saline instead of serum.

‡ 0 = No reaction in mice following injection.

\+ = Ataxia and convulsions.

the inhibiting factor in rabbit serum could be shown to exert a comparable inhibitory effect on the thromboplastic action of tissue suspensions *in vitro*.

Rabbit brain suspensions were tested as thromboplastin by measuring their effect on the clotting time of recalcified rabbit plasma. 0.2 ml. of tissue suspension and 0.2 ml. of 0.025 M $CaCl_2$ were added to 0.2 ml. of oxalated rabbit plasma, at 37°C., and the tubes were observed until coagulation first became detectable. All tests were done in triplicate. By this method, the thromboplastic activity of untreated tissue suspensions was compared with that of suspensions which had been previously mixed and incubated for 1 hour with various dilutions of rabbit serum.

It was found that the *in vitro* method provided little if any evidence of inhibition of the thromboplastic action of rabbit brain suspensions,

except when dilute suspensions of the latter were employed. An illustrative experiment is shown in Table XII, in which are shown the clotting times produced by mixtures of tissue suspensions with serial dilutions of normal rabbit serum, compared with the same suspensions mixed with saline. Also, for comparison, the effect of these suspensions on intravenous injection into mice is shown.

It will be seen that the clotting time produced by a 1–20 dilution of rabbit brain suspension was 17 seconds, and no prolongation of clotting time was observed in this suspension after incubation with rabbit serum. In fact, some enhancement of the thromboplastic effect seemed to occur in the dilutions of serum between 1–4 and 1–128. When the same mixtures were injected into mice, complete inhibition of the toxic action of the tissue suspension was demonstrated in the 1–2, 1–4, and 1–8 dilutions of serum.

When the 1–160 dilution of rabbit brain suspension was employed, the clotting time in the control tube was 22 seconds. Clotting times of 25, 26, and 28 seconds, respectively, were produced by the suspension mixed with the three lower dilutions of serum, suggesting slight inhibitory effect by these dilutions. In contrast, the mixtures of tissue suspension and serum showed complete inhibition in the 1–32 dilution of serum, when tested by injection into mice.

These results indicate that inhibition of the thromboplastic activity of the tissue component under study, which is demonstrable by the *in vivo* effect of this material in mice, is accompanied by very slight, if any, detectable inhibition of thromboplastic activity in recalcified plasma by the method used. The apparent disparity between the two tests may be due in part to the extreme sensitivity of recalcified plasma to small amounts of thromboplastin, and the difficulty in detecting significant time differences within a narrow range in this test. However, other factors may be involved. It is known that fresh serum contains some prothrombin, which might interfere in the *in vitro* test for inhibition. It has also been shown by Maltaner (2) that serum exhibits a thromboplastic effect in recalcified plasma, which becomes enhanced in the presence of tissue thromboplastin. Perhaps this is the basis for the slight enhancement of thromboplastic activity which was observed above in some of the mixtures of tissue suspension and rabbit serum.

DISCUSSION

The property of normal rabbit serum to inhibit the *in vivo* thromboplastic effect of tissue suspensions appears to be due to the combined action of two separate agents. The first of these is a non-dialysable factor, contained within the globulin fraction of serum, and inactivated by heating at 65°C. The second is dialysable, resistant to heating, and consists, in part if not entirely, of calcium. Neither the globulin fraction nor calcium, when used alone, have any demonstrable effect on the tissue component.

The reaction between the serum inhibitor and the tissue component may consist of an actual combination of the two. Evidence which suggests such a combination was obtained in absorption experiments, in which it was found that the titer of serum inhibitor was reduced when the tissue component was added and subsequently removed by centrifugation. Also, the tissue component remained inactive when it was removed from serum and resuspended in saline, but regained almost all of its original activity when oxalate was added. The latter observation indicates that the inhibition reaction is reversible, and may be dependent upon the continued presence of calcium for its maintenance.

Certain analogies may be cited between the inhibition reaction and non-specific complement fixation reactions which are known to occur between normal serum and tissue suspensions. Maltaner (2) has pointed out that a correlation exists between the complement-fixing properties of tissue suspensions and the thromboplastic activity of the same preparations, and has shown that the combination of normal serum and thromboplastic tissue suspensions results in an enhancement of the thromboplastic effect, when the mixtures are added to recalcified plasma. These results are precisely the opposite to the finding of inhibited thromboplastic action which was encountered in the present study by the *in vivo* method. An explanation for the differences between the *in vivo* and *in vitro* reactions is not available. It should be mentioned that in Maltaner's experiments, the mixtures of serum and tissue suspension were tested shortly after being prepared. In the present study, it was found necessary to incubate the mixtures of tissue and serum for 1 hour at 37°C. in order to demonstrate the inhibition reaction.

There are certain similarities between the components which participate in the inhibition reaction, and those which were shown by Kidd and Friedewald (3) to be involved in a complement fixation reaction between normal rabbit serum and mammalian tissue. The tissue component which comprised the antigen in the complement fixation reaction was found by these workers to be entirely sedimentable by high speed centrifugation. The thermostability of centrifuged suspensions was greater than that of crude suspensions, which resembles the observed changes in the thermostability of the tissue component responsible for convulsions and ataxia in mice (1). The serum factor which Kidd and Friedewald described was contained in the globulin fraction, and had the same range of thermostability as the serum factor which is involved in the inhibition reaction. Such similarities are of no more than speculative value, but it is of interest to consider the possibility that the "non-specific" complement fixation reaction and the inhibition reaction described in this paper may be related phenomena.

The actual significance of the inhibiting factor in serum is not known. The uniformity with which it was encountered in all of the rabbits studied, and the constancy of the levels in different sera which were shown by serum titrations, suggest that it may be in some fashion involved in the normal coagulation mechanism. However, there is insufficient evidence to indicate the nature of its role.

The present paper is confined to observations on the inhibiting property of normal rabbit serum. It should be mentioned that a similar factor has been found in other varieties of mammalian serum, including human serum. For practical purposes, the most satisfactory method for determining the amount of inhibiting factor appears to be one which involves the use of heat-inactivated homologous serum as diluent, or in which calcium in excess is added to the higher dilutions of serum tested. By this method, an inhibitor of the sedimentable component of human brain tissue suspensions has been demonstrable in normal human serum, in dilutions as high as 1–128 or 1–256.

SUMMARY

The toxic reaction resulting from intravenous injections of a thromboplastic sedimentable component of rabbit brain tissue, in mice, was prevented by previous incubation of the tissue suspension with normal

rabbit serum. The factor in serum which caused inhibition of the tissue component was not affected by heating for 30 minutes at 56°C., was reduced in activity at 60°C., and completely inactivated at 65°C. It was contained within the globulin fraction of serum.

The presence of calcium was necessary for the inhibitory reaction to take place. Oxalated rabbit plasma showed no inhibitory property. The addition of oxalate to serum prevented inhibition from taking place, and the subsequent addition of calcium caused the restoration of inhibitory action. Dialysis of serum against physiological saline removed the inhibitory property, and the addition of calcium restored it. The addition of calcium to diluted serum caused an enhancement of the inhibitory effect. Calcium alone, without serum, had no effect on the toxicity of tissue suspensions.

Serum which had lost its inhibitory property through dialysis could be restored to activity by mixing with serum which had been inactivated by heating at 65°C.

The inhibitory property in serum was partially removed by absorption with the thromboplastic tissue component. The tissue component remained inactive after removal from serum, but regained its activity when oxalate was added.

The available evidence indicates that the serum factor which inhibits the thromboplastic action of brain tissue suspensions is composed of two portions, one a thermolabile globulin component, and the other a thermostable, dialysable component, which is composed, at least in part, of calcium.

REFERENCES

1. Thomas, L.: Bull. Johns Hopkins Hospital **81:** 1, 1947.
2. Maltaner, F.: Proc. Soc. Exp. Biol. and Med. **62:** 302, 1946.
3. Kidd, J. G., and Friedewald, W. F.: J. Exp. Med. **76:** 543, 1942.

POLYMYXIN: A NEW CHEMOTHERAPEUTIC AGENT

P. G. STANSLY, R. G. SHEPHERD AND H. J. WHITE

Chemotherapy Division, Stamford Research Laboratories of American Cyanamid Company, Stamford, Connecticut

In the course of a program designed to find new antibiotic substances for the chemotherapy of gram-negative bacterial infections, an organism was isolated from soil which produced on an agar plate a wide zone of inhibition of the gram-negative pathogen, *Salmonella schottmuelleri*. The antibiotic-producing organism has been identified as *Bacillus polymyxa* and the antibiotic substance accordingly designated "Polymyxin."

Polymyxin was isolated from the cell-free fermentation liquor as a white, water-soluble hydrochloride. It is unique in its remarkable specificity for gram-negative bacteria thus distinguishing it from all antibiotics hitherto reported. This distinction is further supported by certain chemical and physical properties of the isolated concentrates.

In experimental infections produced by gram-negative bacteria in mice, polymyxin is a highly effective chemotherapeutic agent with a very favorable ratio of therapeutic to acute lethal dose. Of particular interest with respect to chemotherapeutic application is the fact that from a variety of sensitive species, strains resistant to polymyxin have not been obtained under conditions which readily yield strains completely resistant to streptomycin.

The present communication summarizes the main results concerning polymyxin which have been obtained during the course of the past several years. Detailed reports on the various aspects of this work are in preparation.

Identification of Bacillus polymyxa. The identification of this organism was aided greatly by following the key to the identification of aerobic spore-forming bacteria by Smith, Gordon and Clark (1).

Briefly, the following observations characterized the antibiotic-producing organism and established its identity as *Bacillus polymyxa*. Broth cultures 18 hours old revealed gram-negative rods with few or no gram-positive cells. Older cultures showed vegetative cells and

oval spores either free or central to terminal in adhering and swollen sporangia. Carbohydrates such as dextrose, lactose and sucrose were fermented with the formation of both acid and gas. Under the conditions recommended for the respective tests (1), the organism produced acetylmethyl carbinol but no amylase that could catalyze the formation of crystalline dextrins from starch (2).

Potency Assay. The unit of activity is equivalent to the amount of polymyxin per milliliter in Trypticase Soy nutrient agar (Baltimore Biological Laboratory) which just inhibits the growth of the test organism (*Escherichia coli*, MacLeod strain). A filter paper disc agar diffusion method of assay similar to that commonly used for the assay of penicillin or streptomycin but utilizing the above organism has been devised. Under suitable conditions, the method gives a straight line relationship between log concentration and zone diameter over a range of at least 1 to 256 units per milliliter.

Important factors in the assay are the time and temperature of incubation. Solution-saturated discs are placed on seeded agar plates and incubated for 16–18 hours at 25°C. followed by 6 hours at 37° C. Another factor which increased the sensitivity of the assay and the definition of the inhibition zones was the pH of the antibiotic solutions. As a routine measure assays are performed with solutions at pH 2 in .05 M glycine buffer. Buffer alone at this pH gives no inhibition zone. Standard solutions of the antibiotic in glycine buffer at pH 2 were found to be stable indefinitely in the refrigerator.

The method for estimating potency is essentially that given by Knudsen and Randall for penicillin (3). As routinely performed, the error of the assay is approximately $\pm 15\%$ for a 95% probability.

Production. Polymyxin occurs in the cell-free fermentation liquor after cultivation of *Bacillus polymyxa* for 2 to 5 days at 20–30°C. in a neutral medium consisting of 1% glucose, 2% ammonium sulfate, 0.5% yeast extract, 0.2% potassium dihydrogen phosphate, 0.05% magnesium sulfate heptahydrate, 0.001% ferrous sulfate heptahydrate and 0.005% sodium chloride.

Stationary production is satisfactory in shallow layers, e.g. 100 milliliters per 500-milliliter Erlenmeyer flask. Deep fermentation requires moderate aeration. The rate of aeration used for routine production on a laboratory scale (15 liters of medium per 22-liter

round-bottom flask) was approximately 1 liter of air per minute per 15 liters of medium. One milliliter of 1% octadecanol in mineral oil per liter of medium satisfactorily controlled foaming. Deep fermentation ordinarily yields liquor having a potency of about 200 units per milliliter. The yield from shallow layer production is somewhat less.

Isolation of the Active Principle. The clarified 5-day fermentation liquor is treated with a minimum quantity of charcoal (0.4 gram of Darco G-60 per 100 milliliters) to adsorb all the activity. The charcoal is washed successively with water, neutral 50% aqueous ethanol and absolute methanol. The activity is eluted with acidified methanol yielding about 30–50% of the activity of the fermentation liquor. The active principle is then quantitatively precipitated by addition of the eluate to 5 to 10 volumes of acetone. The resulting white powder has a potency of about 1200 units per milligram. Purification of this concentrate through the water-insoluble picrate has resulted in hydrochloride having a potency of about 1800 units per milligram.

Characteristics of the Isolated Concentrate. The material obtained as described above is a white, highly water-soluble hydrochloride whose aqueous solutions have a pH of about 5. The aqueous solubility remains high throughout the pH range 1–11. This salt is very soluble in methanol and shows a rapidly decreasing solubility in higher alcohols. It is insoluble in ether, acetone, chlorinated solvents and the hydrocarbons. The activity of polymyxin as determined by the standard assay is relatively stable to heat in aqueous solutions in the pH range 2–7 and in dilute acidic alcohol solutions. However, the activity is destroyed by strong acid or alkali at room temperature in less than 36 hours. The assay titer of a solution of the isolated concentrate is unaffected by prolonged incubation with pepsin, trypsin, pancreatin and erepsin. The activity passes through a cellophane membrane when dialyzed at 10°C. in aqueous solution at pH 7.

Material having a potency of about 1800 units per milligram is an amorphous white powder, is levo-rotatory in aqueous solution and does not give the Sakaguchi reaction. No characteristic absorption in the ultraviolet is observed in the 240–400 mμ region. The absorption spectrum of this concentrate in the infra-red differs from the spectra of

the known antibiotics such as streptomycin, streptothricin, subtilin, gramicidin-S, tyrocidin and bacitracin.[1]

Preparations of the above potency on treatment in aqueous solution at room temperature with formaldehyde yield a white precipitate upon alkalinization. The resulting precipitate on warming with sodium bisulfite dissolves to form a colorless solution. This solution exhibits all the original activity when assayed at pH 7 (the prevailing pH) or by the standard assay procedure after acidification to pH 2. From a solution prepared in this way, the modified active material can be isolated by vacuum freeze-drying as a white, water-soluble, optically-active powder having no melting point. This derivative retains the high order of activity of the hydrochloride both *in vitro* and *in vivo*.

Antibacterial Activity In Vitro. When tested by the agar streak plate method (4), using 2% blood infusion agar, 56 out of 64 strains of gram-negative bacteria were found to be sensitive to from 0.5 to 16 micrograms of polymyxin hydrochloride[2] per milliliter of test medium. This group of polymyxin-sensitive organisms included the following species and number of strains: *Aerobacter aerogenes*, 3; *Brucella abortus*, 2; *Eberthella typhosa*, 4; *Escherichia coli*, 3; *Hemophilus influenzae*, 1; *Klebsiella pneumoniae*, 9; *Neisseria intracellularis*, 1; *Pasteurella multocida*, 7; *Proteus vulgaris*,[3] 1; *Pseudomonas aeruginosa*, 3; *Salmonella cholerae suis*, 1; *Salmonella enteritidis*, 2; *Salmonella paratyphi*, 1; *Salmonella pullorum*, 3; *Salmonella schottmuelleri*, 2; *Shigella dysenteriae*, 2; *Shigella paradysenteriae*, 7; *Shigella sonnei*, 2; *Shigella gallinarum*, 1; *Vibrio cholerae*,[3] 1. In a few species, variation in the sensitivity of different strains was observed. Thus, of 8 *Pasteurella multocida* strains tested, 7 were sensitive to 2 and 1 was

[1] We are greatly indebted to Dr. R. C. Gore of the Physics Division for the determination and interpretation of the infra-red spectrum.

[2] Concentrations and doses of polymyxin hydrochloride are expressed on the basis of an arbitrarily assigned potency of 2000 units per milligram for the pure salt. Preparations varying from 200 to 1800 units per milligram gave the same biological response per unit.

[3] *Proteus vulgaris* and *Vibrio cholerae* strains were tested in 2% peptone broth, the *Clostridium* strain in thioglycollate medium and the *Mycobacterium* strain in Dubos medium.

sensitive to 64 micrograms per milliliter. In several cases, very little variation in the sensitivity of different strains was noted. As an example of this, the 11 strains of dysentery bacilli, mentioned above, were all found to be sensitive to from 0.5 to 2 micrograms per milliliter.

Under similar test conditions, 34 strains of gram-positive bacteria were resistant to 64 micrograms of polymyxin per milliliter. These polymyxin-resistant organisms represented the following species and number of strains: *Bacillus mycoides*, 1; *Bacillus subtilis*, 1; *Clostridium welchii*,[3] 1; *Corynebacterium diphtheriae*, 4; *Diplococcus pneumoniae*, 1; *Erysipelothrix rhusiopathiae*, 1; *Mycobacterium tuberculosis*,[3] 1; *Staphylococcus aureus*, 1; *Streptococcus hemolyticus*, 20; *Streptococcus agalactiae*, 1; *Streptococcus faecalis*, 1; *Streptococcus viridans*, 1.

The activity of polymyxin was not affected significantly by variations in the composition of test medium such as the addition of defibrinated rabbit blood up to 50% or by changes in the pH of the medium ranging from pH 5 to 8.

In a study to determine whether resistant organisms could be selected from large populations of cells (10 billion, or more) of each of 15 normally sensitive strains, representing 8 pathogenic gram-negative genera, we have thus far failed to obtain any organisms with an appreciable degree of resistance to polymyxin. Under the same conditions, organisms which for all practical purposes are completely resistant to streptomycin can be readily obtained from most of these strains. Preliminary results of this study are given in Table I.

Therapeutic Activity. Polymyxin was found to be highly effective when administered subcutaneously as a single dose to mice infected with virulent strains of *Klebsiella pneumoniae* or *Pasteurella multocida*. In experimental infections produced by each of 5 highly virulent strains of *Klebsiella*, a single dose of 10 micrograms per 20 gram mouse sufficed to protect 80–100% of the animals (Table II). Under similar test conditions, a dose of 40 micrograms was similarly effective in experimental infections produced by each of 5 strains of *Pasteurella* (Table III). Pooled results of several therapeutic experiments using *Klebsiella pneumoniae*, strain BE, and *Pasteurella multocida*, strain 310, are given in Tables IV and V. From these tables it is evident that the Median Survival Doses ($S.D._{50}$'s) for the *Klebsiella* and the *Pasteurella* infections are approximately 0.4 and 1.0 milligrams per

kilogram, respectively. Polymyxin was found to be equally effective, whether given immediately or 4 hours after infection.

TABLE I

Selection of Drug-Resistant Bacteria from Large Populations of Normally Sensitive Strains

TEST ORGANISM AND STRAIN*	AGAR POUR PLATE		NUMBER OF COLONIES§ RESISTANT TO	
	Number† of Bacteria	Concentration‡ of Antibiotic	Streptomycin	Polymyxin
	Billions	*μg./ml.*		
A. aerogenes (EF)	26	160	3	0
A. aerogenes (EF)	25	1280	11	
E. typhosa (58)	14	160	220	0
E. coli (MK)	11	160	7	0
E. coli (MK)	20	1280	11	
E. coli (MacLeod)	35	160	2	0
K. pneumoniae (CH)	19	160	3	0
K. pneumoniae (CH)	30	1280	5	
K. pneumoniae (CHA)	22	160	12	0
K. pneumoniae (CHA)	42	1280	13	
K. pneumoniae (BE)	18	160	0	0
Paracolon (WT)	19	160	2	0
P. multocida (310)	10	160	10	0
P. aeruginosa (CM)	55	160	81	0
P. aeruginosa (CM)	26	1280	45	
S. gallinarum (469)	28	160	0	0
S. paratyphi (Stam.)	17	160	>1000	0
S. pullorum (Hol.)	18	160	0	0
S. schottmuelleri (Merck)	28	160	3	0
S. flexner (5733)	17	160	0	0

* Each of the strains listed above was sensitive to from 2 to 16 micrograms of either streptomycin or polymyxin per milliliter of medium when tested by conventional methods using an inoculum size of approximately 1,000,000 cells. Strains CH, CHA, EF, MK, CM and WT were kindly supplied by Dr. Maxwell Finland.

† Infusion agar pour plates containing each drug were seeded with the indicated number of cells, as determined by colony counts of appropriate dilutions in control plates.

‡ Drug concentrations are expressed as pure base for streptomycin and as pure hydrochloride for polymyxin (see footnote 2).

§ Colonies which appeared on these plates during incubation at 37°C. for 48 hours were subcultured into broth. Relatively small inocula of the strains thus recovered were found to be resistant to 1280 μg. of streptomycin per milliliter.

When the severity of the *Klebsiella* infection was increased by inoculation with 140,000 and with 14,000,000 lethal doses, the dose of polymyxin required for 90–100% survival was 3.2 and 6.4 milligrams per kilogram, respectively (Table VI).

TABLE II

Therapeutic Activity of Polymyxin Hydrochloride Against Five Strains of Klebsiella pneumoniae in Mice

Mice: Vanderwerken; 16–24 grams.

Infections: Intraperitoneal; 0.5 cc. of 10^{-6} dilution of 5-hour broth cultures; 600–1600 lethal doses.

Treatment: Subcutaneous; single dose given immediately after infection; aqueous solution of drug at pH 7.0.

DOSAGE*	NUMBER OF MICE PER DOSE	NUMBER OF MICE ALIVE ON 21ST DAY AFTER INFECTION				
		Strain of Klebsiella				
		A-R_u	A-S_e	B-B_R	B-E_G	C-F
mg./kg.						
4.0	10	10	10	10	10	8
2.0	10	10	10	10	10	9
1.0	10	10	9	10	8	9
0.5	10	10	10	8	10	10
0.25	10	2	8	1	10	6
Untreated	10	0	0	0	0	0

* Dosage expressed as pure polymyxin hydrochloride. See footnote 2.

TABLE III

Therapeutic Activity of Polymyxin Hydrochloride Against Five Strains of Pasteurella multocida in Mice

Mice: Vanderwerken; 16–24 grams.

Infections: Intraperitoneal; 0.5 cc. of 10^{-6} dilution of broth cultures; 200–800 lethal doses.

Treatment: Subcutaneous; single dose given immediately after infection; aqueous solutions of drug at pH 7.0.

DOSAGE*	NUMBER OF MICE PER DOSE	NUMBER OF MICE ALIVE ON 21ST DAY AFTER INFECTION				
		Strain of Pasteurella				
		31	53	221	310	398
mg./kg.						
16	10	10	10	10	10	10
8	10	10	10	10	10	9
4	10	10	10	10	9	10
2	10	8	10	9	8	9
1	10	4	7	6	2	2
Untreated	10	0	0	0	0	0

* Dosage expressed as pure polymyxin hydrochloride. See footnote 2.

TABLE IV

Therapeutic Activity of Polymyxin Hydrochloride Against Klebsiella pneumoniae, strain BE, in Mice

Mice: Vanderwerken; 16–24 grams.

Infection: Intraperitoneal; 0.5 cc. of 10^{-6} dilution of a 4-hour broth culture; 5000 ± 2000 organisms.

Treatment: Subcutaneous; single dose given immediately after infection; aqueous solutions of drug at pH 7.0 ± 0.2.

DOSAGE*	SURVIVAL—21 DAYS AFTER INFECTION		
mg./kg.	*Alive/Total*	*Per cent*	*Time†*
4.0	70/70	100	
2.0	70/70	100	
1.0	70/70	100	
0.8	76/80	95	4.5
0.6	69/80	86	5.6
0.4	40/80	50	3.8
0.2	0/80	0	1.7
Untreated	0/80	0	1.3

* Dosage expressed as pure polymyxin hydrochloride. See Footnote 2.

† Average survival time (days) for mice that died.

TABLE V

Therapeutic Activity of Polymyxin Hydrochloride Against Pasteurella multocida, strain #310, in Mice

Mice: Vanderwerken; 16–24 grams.

Infection: Intraperitoneal; 0.5 cc. of 10^{-6} dilution of a 5-hour blood broth culture; 500 ± 100 organisms.

Treatment: Subcutaneous; single dose given immediately after infection; aqueous solutions of drug at pH 7.0 ± 0.2.

DOSAGE*	SURVIVAL—21 DAYS AFTER INFECTION		
mg./kg.	*Alive/Total*	*Per cent*	*Time†*
16.0	30/30	100	
8.0	30/30	100	
4.0	55/60	92	1.8
2.0	43/60	72	1.4
1.0	27/60	45	1.2
0.6	20/60	33	1.1
0.2	0/30	0	1.1
Untreated	0/60	0	1.1

* Dosage expressed as pure polymyxin hydrochloride. See footnote 2.

† Average survival time (days) for mice that died.

Polymyxin is equally effective whether administered subcutaneously or intravenously; oral administration required about 64 times as much drug to obtain the same therapeutic response (Table VII).

Sixteen milligrams of polymyxin per kilogram of body weight, when administered once daily for 4 days, protected two week old chickens from an otherwise fatal infection with a strain of fowl typhoid (Table VIII).

TABLE VI

The Influence of Size of Infecting Dose on the Therapeutic Effectiveness of Polymyxin Hydrochloride

Organism: Klebsiella pneumoniae; strain B-E.

Mice: Vanderwerken; 16–24 grams.

Infections: Intraperitoneal; 0.5 cc. of 10^{-3}, 10^{-4} and 10^{-5} dilutions of a 4-hour broth culture.

Treatment: Subcutaneous; single dose immediately after infection; aqueous solutions at pH 7.0 ± 0.2.

DOSAGE*	NO. OF MICE PER DOSE	NUMBER OF MICE ALIVE ON 21ST DAY AFTER INFECTION		
		1400†	140,000†	14,000,000†
mg./kg.				
6.4	10			9
3.2	10		9	6
1.6	10	10	8	5
0.8	10	8	2	3
0.4	10	3	0	1
0.2	10	0	0	
0.1	10	0		
Untreated	10	0	0	0

* Dosage expressed as pure polymyxin hydrochloride. See footnote 2.

† Number of organisms in infecting dose.

Toxicity. Polymyxin preparations do not exhibit histamine-like activity. When administered subcutaneously to mice, the $L.D._{50}$ of the hydrochloride[2] was found to be approximately 300 milligrams per kilogram of body weight. Injection of 0.75% solutions of this salt caused local skin irritation. The formaldehyde bisulfite derivative, however, caused no local irritation even when injected as a 15% solution. Furthermore, this derivative was less toxic acutely than the hydrochloride. Preliminary chronic toxicity studies with this derivative have revealed no evidence of toxicity in young rats when

TABLE VII

Comparative Effectiveness of Polymyxin Hydrochloride Administered by Different Routes

Organism: Klebsiella pneumoniae; strain B-E.
Mice: Vanderwerken; 16–24 grams.
Infection: Intraperitoneal; 0.5 cc. of 10^{-6} dilution of 4-hour broth culture; 5000 organisms.
Treatment: Route indicated below; single dose immediately after infection; aqueous solutions at pH 7.0 ± 0.2.

DOSAGE*	NO. OF MICE PER DOSE	NUMBER OF MICE ALIVE ON 21ST DAY AFTER INFECTION		
		Oral	Subcutaneous	Intravenous
mg./kg.				
102.4	10	10		
51.2	10	9		
25.6	10	0		
12.8	10	0		
6.4	10	0		
3.2	10	0	10	10
1.6	10		10	10
0.8	10		10	10
0.4	10		9	5
0.2	10		0	0

* Dosage expressed as pure polymyxin hydrochloride. See footnote 2.

TABLE VIII

Therapeutic Activity of Polymyxin Hydrochloride in an Experimental Fowl Typhoid Infection

Organism: Shigella gallinarum; strain #469.
Birds: 2-week old chicks.
Infection: Intravenous; 0.25 cc. of 10^{-1} dilution of a 5-hour broth culture.
Treatment: Subcutaneous; 0.5 cc. of aqueous solutions of drug; immediately after infection, then once daily for 4 days.

DOSAGE*	NUMBER OF BIRDS PER DOSE	SURVIVAL—21 DAYS AFTER INFECTION	
		Alive /Total	Survival Time†
mg./kg./day			
16	10	10/10	
8	10	7/10	8.0
4	10	1/10	4.4
Untreated	10	0/10	3.4

* Dosage expressed as pure polymyxin hydrochloride. See footnote 2.
† Survival time (days) for birds that died.

doses of 100 milligams per kilogram per day were administered subcutaneously for thirty days.

SUMMARY

A new chemotherapeutic agent designated "Polymyxin" has been isolated as the hydrochloride from the fermentation liquor of *Bacillus polymyxa*. The production, isolation and preliminary characterization of this antibiotic have been described and a method for its assay outlined.

Polymyxin is specific for gram-negative bacteria. It is therapeutically active in experimental infections in mice and relatively non-toxic.

From a variety of sensitive species, strains resistant to polymyxin have not been obtained under conditions which readily yield strains completely resistant to streptomycin.

Acknowledgment: Valuable contributions to various aspects of the work on polymyxin have been made by the following members of the Chemotherapy Division: isolation of *Bacillus polymyxa* and preliminary characterization of the antibiotic, Miss M. E. Schlosser; identification of *Bacillus polymyxa*, Misses N. H. Ananenko, M. H. Cook and Dr. W. C. Tobie; assay method, Miss M. E. Schlosser; assays, Misses N. H. Ananenko, M. H. Cook and M. E. Schlosser; production and isolation, Misses N. H. Ananenko, M. H. Cook, C. E. Fellows, G. Guillet and M. E. Schlosser; *in vitro* and *in vivo* studies, Miss C. Alverson, Miss M. J. Baker, Mrs. A. H. Clapp, Mrs. M. L. Colborn, Mrs. E. R. Jackson; bacterial resistance studies, Mrs. A. H. Clapp; toxicity studies, Drs. J. T. Litchfield, Jr. and E. J. Robinson and Miss B. C. Wadsworth; purification and chemical studies, Drs. J. P. English and R. Winterbottom, Misses C. E. Fellows, E. B. Leffler and M. M. Rogers.

ADDENDUM

Shortly after submission of the present report for publication, a paper reporting the independent discovery of the antibiotic activity of *Bacillus polymyxa* was presented at the the Annual Meeting of the Society of American Bacteriologists, in Philadelphia, on May 16,

1947, by R. G. Benedict and A. F. Langlykke of the Northern Regional Research Laboratory, Peoria, Illinois.

REFERENCES

(1) SMITH, N. R., GORDON, R. E., AND CLARK, F. E.: Aerobic Mesophilic Spore-forming Bacteria. Misc. Publication No. 559, U. S. Dept. Agr., May, 1946.

(2) TILDEN, E. B. AND HUDSON, C. S.: Preparation and Properties of the Amylases Produced by Bacillus Macerans and Bacillus Polymyxa. J. Bact., **43:** 527, 1942.

(3) KNUDSEN, L. F. AND RANDALL, W. A.: Penicillin Assay and its Control Chart Analysis. J. Bact., **50:** 187, 1945.

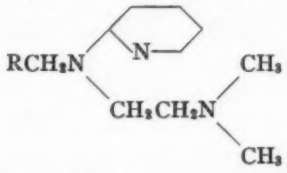
(4) WAKSMAN, S. A., AND REILLY, H. C.: Agar-Streak Method for Assaying Antibiotic Substances. Ind. Eng. Chem., Anal. Ed., **17:** 556, 1945.

5-HALO-2-THENYL DERIVATIVES OF N,N-DIMETHYL-N′-2-PYRIDYL-ETHYLENEDIAMINE AS ANTIHISTAMINICS

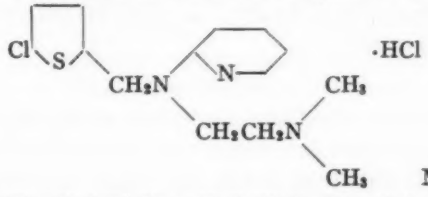
J. T. LITCHFIELD, JR., MAXINE R. ADAMS, LOUISE GODDARD, MARION S. JAEGER AND LILLIAN ALONSO

Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Connecticut

In the course of a study of derivatives of ethylenediamine of the type

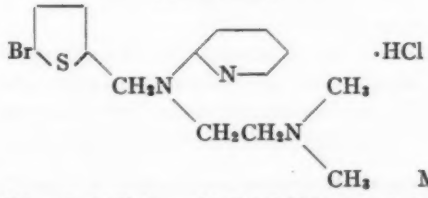


where R is a heterocyclic radical, two compounds synthesized in the Chemotherapy Division were found to possess sufficient antihistamine activity to merit extensive investigation. The structural formulae, melting points and names of these two compounds are given below:



M.P. 106-108°C

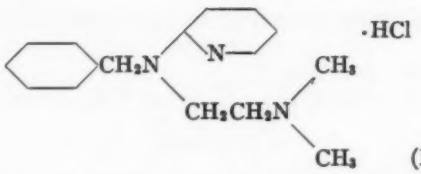
N,N-Dimethyl-N′-(2-pyridyl)-N′-(5-chloro-2-thenyl)-ethylenediamine hydrochloride
(Chlorothen)



M.P. 123-124°C.

N,N-Dimethyl-N′-(2-pyridyl)-N′-(5-bromo-2-thenyl)-ethylenediamine hydrochloride
(Bromothen)

The synthesis and chemical properties of this series of compounds will be described in a forthcoming publication (1). For purposes of convenience the two compounds above will be referred to as Chlorothen and Bromothen, respectively. It was felt desirable, in studying these compounds, to evaluate them with reference to an established antihistamine agent of the ethylenediamine type. For this purpose N,N-dimethyl-N′-(2-pyridyl)-N′-(benzyl)-ethylenediamine hydrochloride,[1]



(Pyribenzamine),

was selected as a reference standard, since considerable experimental data on its pharmacological properties were available (2–8).

Results of studies made thus far indicate that Chlorothen and Bromothen are twice as active and one half as toxic acutely as Pyribenzamine when compared on a weight basis. When equal doses of the drugs are administered, Chlorothen and Bromothen protect twice as long as Pyribenzamine.

METHODS

The experimental techniques used were essentially the same as those reported by other investigators in this field (2–9). In tests utilizing isolated guinea pig ileum, the degree of inhibition of the response to 0.04 micrograms of histamine diphosphate per cc. or 0.02 micrograms of acetylcholine chloride per cc. was studied. When the drugs were examined for activity in guinea pigs, survival or death was found to be the most reliable response.

Histamine aerosols were generated from a 1.25 per cent histamine diphosphate solution. This was found to be nine times the median lethal concentration under our conditions. For intravenous histamine shock, 1 mgm. of histamine (about four times the $L.D._{50}$) was injected

[1] We are indebted to the Pharmaceutical Group of the Calco Chemical Division of American Cyanamid Company, for supplying this compound for use in these studies.

into the foreleg vein of the guinea pigs. Control mortality rates based on 50 or more animals were 95 per cent for histamine aerosol, and 100 per cent for intravenous histamine. The number of guinea pigs used for each drug in each test summarized in the tables varied from 40 to 180. Dosage values of all drugs used in these studies are given in terms of the salts and not the free bases. Complete details of these and other experiments in progress will be given in subsequent reports.[2]

RESULTS

Table 1 summarizes the investigations on Chlorothen, Bromothen and Pyribenzamine. In the case of isolated guinea pig ileum, it was found that 5 to 10 minutes exposure to the first two drugs was necessary to develop maximal antihistamine effect[3]. It was found also that the recovery time after exposure to Chlorothen or Bromothen, was 4 to 5 times as long as that for Pyribenzamine. This comparison was made by measuring for each drug, the half-recovery time from equal degrees of inhibition of the histamine stimulus. On the basis of the concentration giving 50 per cent inhibition of histamine Chlorothen and Bromothen were found to be 4 times more active than Pyribenzamine, as is shown by the inhibitory ratios in table 1. However, the three drugs were found to be relatively inactive against acetylcholine.

Using histamine aerosol Chlorothen and Bromothen were compared to Pyribenzamine for activity after oral as well as intraperitoneal administration. In these experiments the percentage of guinea pigs protected (from death) was determined for graded doses of each compound.

[2] We are indebted to Walton Marsh for assistance in standardizing techniques for evaluating antihistamine activity, and to Dr. R. L. Burkhart of the Animal Industry Section of Lederle Laboratories Division of American Cyanamid Company, for furnishing most of the guinea pigs used in these studies.

[3] Chlorothen and Bromothen have physical properties such that they deposit as an oily film especially on any rough glass surface if the pH of 0.01 to 0.1 per cent aqueous solutions is raised above 7.0 Such a film of drug usually cannot be removed by washing with distilled water or Tyrodes solution and may introduce serious error into bioassay and analytical procedures. As a consequence alkaline detergents are not satisfactory for cleaning glassware which has been in contact with these drugs unless the glassware is rinsed also with N/10 HCl and finally with distilled water.

By plotting these data, the Median Protective Dose ($P.D._{50}$) could be estimated (10). In a similar manner the drugs were compared for protective activity against intravenous histamine. The $P.D._{50}$ values given in table 1 show that Chlorothen and Bromothen are at least twice as active as Pyribenzamine in guinea pigs.

TABLE 1

Activity and Toxicity of Antihistominics

NATURE OF TEST	DEFINITION OF ENDPOINT		CHLOROTHEN	BROMOTHEN	PYRIBENZAMINE
Isolated Guinea Pig Intestine	Histamine Conc. / Inhibitor Conc.		100	100	25
	Acetylcholine Conc. / Inhibitor Conc.		0.01 to 0.001	0.01 to 0.001	0.01 to 0.001
Lethal Histamine Aerosol	$P.D._{50} \pm$ S.E.*	I.P.§	0.22 ± 0.06	0.25 ± 0.04	0.72 ± 0.10
	mgm./Kgm.	P.O.‖	2.04 ± 0.45	1.76 ± 0.32	3.54 ± 0.82
Lethal Intravenous Histamine	$P.D._{50} \pm$ S.E.	I.P.	0.10 ± 0.02	0.11 ± 0.02	0.27 ± 0.05
			Experiment 1	*Experiment 2*	
Lethal Histamine Aerosol	P.T.$_{50}$ in hours† after oral dosage	10 mgm./Kgm.	8.0	7.5	9.0 4.5
		5 mgm./Kgm.	6.2	7.0	5.2 2.1
		2.5 mgm./Kgm.	3.2	3.8	2.6 1.4
		1.25 mgm./Kgm.	2.5	2.5	0.8 <0.5
Acute Toxicity in Mice	$L.D._{50} \pm$ S.E.‡	P.O.	390 ± 27	430 ± 25	200 ± 14
	mgm./Kgm.	I.P.	112 ± 10	130 ± 6	62 ± 7
Acute Toxicity in Rabbits	$L.D._{50} \pm$ S.E.	I.V. ¶	77 ± 14	79 ± 7	27 ± 3

* = Median Protective Dose ± Standard Error.
† = Median Protective Time ± Standard Error.
‡ = Median Lethal Dose ± Standard Error.
§ = Intraperitoneal.
‖ = Per Os.
¶ = Intravenous.

A comparison of these drugs to Pyribenzamine for duration of protection is summarized also in table 1. In these experiments graded doses of the two compounds were administered by mouth and one hour later the guinea pigs were given the standard exposure to histamine aerosol. The survivors were re-exposed at suitable intervals until all had succumbed. By plotting the cumulative mortality against time and fitting the curve, the Median Protection Time, $P.T._{50}$, for each

dose of each drug could be estimated. These $P.T._{.50}$ values which are given in table 1 were also plotted against the corresponding doses and a straight line fitted for each drug. The ratio: slope constant of a halothen to that of Pyribenzamine is the best estimate of the relative activity of the two drugs, with respect to duration of protection. This ratio was 2.0 and it indicates that when equal doses of the drugs were given, the duration of protection was twice as long for Chlorothen and Bromothen as it was for Pyribenzamine.

TABLE 2

Activity and Toxicity of Antihistaminics

NATURE OF TEST	DEFINITION OF ENDPOINT		THENYL D.P.E.	BROMO-BENZYL D.P.E.	PYRIBENZ-AMINE
Isolated Guinea Pig Intestine	Histamine Conc. / Inhibitor Conc.		15	50	25
Lethal Histamine Aerosol‖	$P.D._{50} \pm S.E.$* mgm./Kgm.	I.P.§	1.29 ± 0.21	0.48 ± 0.10	0.35 ± 0.06 / 0.50 ± 0.10
Lethal Intravenous Histamine	$P.D._{50} \pm S.E.$ mgm./Kgm.	I.P.	0.43 ± 0.08	0.25 ± 0.04	0.31 ± 0.06
Lethal Histamine Aerosol	$P.T._{50}$ in hours† after oral dosage	2.5 mgm./Kgm. 1.25 mgm./Kgm.	0.8 <0.5	1.6 0.5	1.4 0.5
Acute Toxicity Mice	$L.D._{50} \pm S.E.$‡ mgm./Kgm.	I.P.	79 ± 12	160 ± 34	62 ± 7

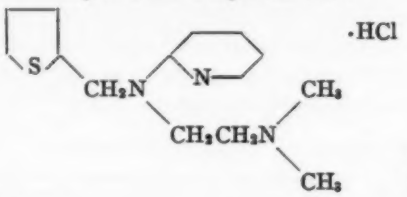
* = Medium Protective Dose ± Standard Error.
† = Median Protective Time ± Standard Error.
‡ = Median Lethal Dose ± Standard Error.
§ = Intraperitoneal.
‖ = These results are comparable to results in table 1, only on a relative basis because a different atomizer was used to produce the histamine aerosol.

The results of studies on the acute oral and intraperitoneal toxicity for mice and intravenous toxicity for rabbits are also summarized in table 1, where it can be seen that the halo-thenyl derivatives were one-half as toxic as Pyribenzamine. In all cases in table 1, where standard errors are given, the difference between the value for a halo-thenyl derivative and the corresponding value for Pyribenzamine exceeded the standard error of the difference by more than two-fold and hence could be regarded as significant.

Table 2 summarizes the data obtained on two related compounds

which are of particular interest. The first of these, which is the parent compound of the thiophene series, is:

N,N-Dimethyl-N′-(2-pyridyl)-N′-(2-thenyl)-ethylenediamine hydrochloride

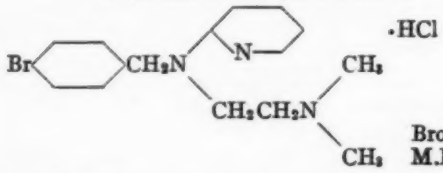


Thenyl D.P.E.
M.P. 162-165°C

The synthesis of this compound has been reported independently by Weston (11).

The second one is:

N,N-Dimethyl-N′-(p-bromobenzyl)-N′-(2-pyridyl)-ethylenediamine hydrochloride



Bromobenzyl D.P.E.
M.P. 184-186°C

The data in table 2 show that the thenyl derivative is less than one third as active as Pyribenzamine against histamine aerosol and similarly toxic acutely in mice. Limited studies on the duration of protection indicate that dose for dose Thenyl D.P.E. gives a shorter period of protection than Pyribenzamine (table 2). The data on the Bromobenzyl D.P.E. indicate that its activity and duration of protection are equal to, but its acute toxicity is one-half that of Pyribenzamine. A comparison of Thenyl D.P.E. and Bromobenzyl D.P.E. with Pyribenzamine for activity against intravenous histamine is also given in table 2 and shows that Thenyl D.P.E. is less active than Bromobenzyl D.P.E. and Pyribenzamine.

RESULTS OF TOXICITY STUDIES

The studies on acute toxicity of this group of related compounds have been summarized in tables 1 and 2. Although highly significant

differences in toxicity were found, in no case did the symptoms of toxicity differ from one drug to another. All drugs behaved as central nervous system stimulants, causing excitement, tremors, convulsions, tonic and clonic muscle spasms, and respiratory irregularities, all of which have been previously described for Pyribenzamine (3) and Benadryl (12).

Additional studies of the acute toxicity of Bromothen and of Pyribenzamine were made in dogs. A healthy mongrel was given 2 mgm./Kgm. doses of Pyribenzamine intravenously at 20-minute intervals. The second dose precipitated an epileptiform type of convulsion lasting about 5 minutes. Thereafter, the animal exhibited restlessness, respiratory irregularities and frequent aimless movements. A second convulsion occurred after the sixth dose and death occurred after a total of 18 mgm./Kgm. had been administered. A second healthy mongrel was given 4 mgm./Kgm. doses of Bromothen intravenously at 20-minute intervals. After the third dose this animal showed a few signs of beginning toxicity such as occasional aimless movements. After the seventh dose, the animal exhibited excitement, restlessness, irregular respiration, and constant, aimless movements. After receiving a total of 36 mgm./Kgm. a severe epileptiform convulsion occurred followed by unconsciousness. Convulsions recurred intermittently during the next hour, after which the animal became conscious. At 24 hours the animal accepted food and at 48 hours appeared completely normal. This same dog, two weeks later, was given 30 mgm./Kgm. of Pyribenzamine by mouth before feeding. Within 30 minutes a convulsion occurred and this was succeeded by a period of violent excitement. The animal became comatose after the first hour and died 2 hours after the dose was given. A third healthy mongrel survived a single dose of 20 mgm./Kgm. of Pyribenzamine by mouth but not 30 mgm./Kgm. Thus the acute lethal oral dose of Pyribenzamine for dogs is in the range of 20 to 30 mgm./Kgm.

One mongrel and one beagle survived a single dose of 90 mgm. Bromothen per Kgm. by mouth. The mongrel died after 120 mgm./Kgm., but the beagle survived. A second beagle died after 120 mgm./Kgm. Thus the acute lethal oral dose of Bromothen is in the range of 90 to 120 mgm./Kgm.

Two male and two female purebred beagles were given 30 mgm./

Kgm. of Bromothen by mouth daily before feeding. About ½ hour after the fifth dose the animals showed mild psychosomatic symptoms of short duration, such as were described for Benadryl (12), and which we have noted also with mildly intoxicating doses of Pyribenzamine. These symptoms recurred and seemed to increase in severity and duration with the sixth and seventh dose. Accordingly, the daily dose was reduced to 20 mgm./Kgm. At this level no significant symptoms were encountered up to 30 days. Salivation after dosage was noted in all four dogs. Vomiting occurred twice in the female pair. Both males developed a conditioned salivary reflex about the 25th day of dosage. After 30 days the two females showed some loss of appetite. Accordingly their daily dose was reduced to 15 mgm./Kgm. The four animals eat and behave normally, and there has been no loss of body weight of any consequence during the first 60 days of drug administration. Hematological studies of the dogs were carried out before therapy and after 15 days. No departure from normal values was found.

Five albino mice (Tumblebrook Farms) ingesting 20 mgm./Kgm./day and ten ingesting 160 mgm./Kgm./day of Bromothen gained weight at the same rate as control mice and appeared normal throughout a 30-day period. Ten weanling rats (Tumblebrook Farms hooded strain) ingesting 65 mgm./Kgm./day and ten ingesting 700 mgm./Kgm./day of Bromothen appeared normal throughout a 30-day period. The growth rate of the first group did not differ from that of the controls. The growth rate of the 700 mgm./Kgm./day rats was depressed, as was their daily food intake, so that by the end of the 30 days their body weight averaged 30 per cent less than the controls. The mice and rats were autopsied at the end of 30 days. No gross pathology was found in any of the animals. Microscopic studies of the tissues will be reported subsequently.[4]

DISCUSSION

It is well known that thiophene is nearly equivalent chemically to benzene and several thiophene analogs of benzene derivatives have been examined for biological activity (13). In Weston's report on the thenyl

[4] We are indebted to Dr. E. Mayer for pathological examination of the experimental animals.

analog of Pyribenzamine (11) the activity and toxicity were stated to be of the order of Pyribenzamine.

The data reported in this communication indicate that the thenyl analog is neither more active nor less toxic than Pyribenzamine and suggest the compound to be somewhat less active.

Halogenation of the para position of the benzyl group of Pyribenzamine appears to decrease toxicity without significantly affecting activity. On the other hand, introduction of a methoxy group in the para position definitely enhances activity but does not decrease toxicity (9). The effect of halogen in the case of Bromothen and Chlorothen is truly remarkable, since activity is increased four-fold and acute toxicity halved over that of the non-halogenated compound. The acute and chronic toxicity studies of Bromothen in dogs indicate that this compound is well tolerated in doses greatly exceeding those presumably necessary for therapy in man. The long duration of action of Chlorothen and Bromothen suggests that one dose per day may be sufficient in most cases to protect from histamine-type reactions which are encountered in various allergic conditions. However, clinical studies of Chlorothen and Bromothen will be necessary to establish the value of these new antihistaminics.

SUMMARY

Four ethylenediamine derivatives were studied for antihistamine activity in comparison with Pyribenzamine, N,N-Dimethyl-N′-benzyl-N′-(2-pyridyl)-ethylenediamine hydrochloride. It was found that replacement of the benzyl by a thenyl group decreased activity without changing toxicity. Replacement of benzyl by 5-bromo-2-thenyl or 5-chloro-2-thenyl increased activity and decreased toxicity. Replacement of benzyl by para-bromobenzyl decreased toxicity but did not significantly change the activity. The bromothenyl derivative of dimethyl-pyridyl-ethylenediamine was found to be well tolerated over a 30-day period by mice and rats and a 60-day period by dogs, in doses greatly exceeding those showing antihistamine activity in guinea pigs.

REFERENCES

(1) Clapp, Clark, Vaughan, English and Anderson: J. Am. Chem. Soc., 69: 1549, 1947.

(2) MAYER: J. Allergy, **17:** 153, 1946.
(3) MAYER, HAYS, BROUSSEAU, MATHIESON, RENNICK AND YONKMAN: J. Lab. and Clin. Med., **31:** 749, 1946.
(4) YONKMAN, CHESS, MATHIESON AND HANSEN: J. Pharm. & Exper. Therap., **87:** 256, 1946.
(5) MAYER AND BROUSSEAU: Proc. Soc. Exper. Biol. & Med., **63:** 187, 1946.
(6) YONKMAN, OPPENHEIMER, RENNICK AND PELLET: J. Pharm. & Exper. Therap., **89:** 31, 1947.
(7) MARSH AND DAVIS: J. Pharm. & Exper. Therap., **89:** 234, 1947.
(8) SHERROD, LOEW AND SCHLOEMER: J. Pharm. & Exper. Therap., **89:** 247, 1947.
(9) LOEW, KAISER AND MOORE: J. Pharm. & Exper. Therap., **83:** 120, 1945.
(10) LITCHFIELD AND FERTIG: Bull. Johns Hopkins Hosp., **69:** 276, 1941.
(11) WESTON: J. Am. Chem. Soc., **69:** 980, (April) 1947.
(12) GRUHZIT AND FISKEN: J. Pharm. & Exper. Therap., **89:** 227, 1947.
(13) STEINKOPF: Die Chemie des Thiophens. Reprint Edition published by Edwards Bros., Inc., Ann Arbor, Mich., 1944.

PODOPHYLLOTOXIN

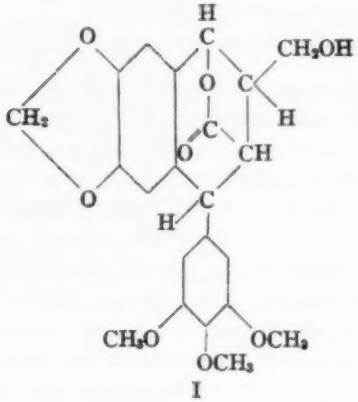
A Preliminary Note

MAURICE SULLIVAN AND KENNETH BLANCHARD

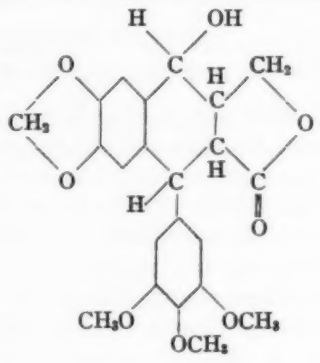
From the Department of Medicine (Dermatology) and the Department of Pharmacology

When podophyllin, the resin of podophyllum peltatum, is applied to condylomata acuminata there is rapid involution of the tumors (1). Histologic studies of human and rabbit skin, condylomata acuminata and verrucae vulgares treated with podophyllin revealed severe cytotoxic effects and distorted mitotic figures similar to those produced by colchicine (2). Furthermore, condylomata acuminata treated with colchicine were rapidly cured. Podophyllin in powder form or made into a paste with water or incorporated in ointments or suspended in oil or dissolved in alcohol produced the aforementioned cytologic changes. However, when podophylln was dissolved in normal solution of potassium or sodium hydroxide it proved to be biologically inert (3). This indicated that podophyllotoxin was probably the ingredient responsible for the involution of the tumors and the cellular alterations because podophyllotoxin is easily susceptible to alkaline hydrolysis. Podophyllin is a mixture of four components: podophyllotoxin, picropodophyllin, podophylloresin and quercetin (4, 5). In dilute solutions of both inorganic and organic bases podophyllotoxin (I) rapidly undergoes rearrangement to the isomeric picropodophyllin (II). More concentrated solutions of inorganic bases convert both of these substances to podophyllic acid (III). The structures of the substances shown below, and the nature of these transformations have been established by the investigations of Borsche (6) and of Spath (7). An alternative structure for podophyllotoxin has been suggested by Robertson and Waters (8). This no longer merits consideration since a substance of this structure upon rearrangement to picropodophyllin and subsequent successive dehydration and dehydrogenation should yield a lactone of established constitution (9) which is isomeric with the lactone obtained by the analogous degradation of podophyllotoxin. Taken together with the observation that alkaline solutions of podophyllin appear devoid of action on the skin, these chemical considerations suggest that this is uniquely determined by the podophyllotoxin content which is stated (5, 10) to account for from 28.2 to 50 per cent of commercial

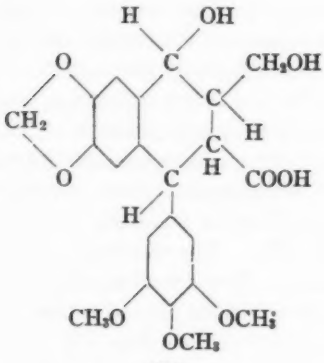
podophyllin. Accordingly, attention was turned to an examination of podophyllotoxin. Podophyllotoxin applied to normal skin and mucous membrane produced gross and microscopic changes identical with



I



II



III

those produced by podophyllin. Condylomata acuminata treated with podophyllotoxin rapidly underwent involution.

CONCLUSION

Podophyllotoxin appears to be the ingredient in podophyllin responsible for its cytologic effects.

We are indebted to Doctor Milton Bush for a commercial preparation of podophyllotoxin.

BIBLIOGRAPHY

1. KAPLAN, I.: New Orleans Med. & Surg. J. **94:** 338, 1942.
2. KING, L. S., AND SULLIVAN, M.: Science **104:** 244, 1946.
3. SULLIVAN, M., AND KING, L. S.: Arch. Derm. and Syph. (In press).
4. U. S. Dispensatory. XX, p. 879.
5. VIEHOEVER, A., AND MACK, H.: J. Am. Pharm. Assoc. **27:** 632, 1938.
6. BORSCHE, W., AND NIEMANN, J.: Ann. **499:** 59, 1932.
7. SPATH, E., WESSELY, F., AND NADLER, E.: Ber. **66:** 125, 1933.
8. ROBERTSON, A., AND WATERS, R. B.: J. Chem. Soc. 83, 1933.
9. HAWORTH, R. D., RICHARDSON, T., AND SHELDRIC, G.: J. Chem. Soc. 1576–1581, 1935.
10. WARREN, L. E.: J. Assoc. Official Agr. Chem. **13:** 117, 1930.

PROCEEDINGS OF THE MEETINGS OF THE JOHNS HOPKINS MEDICAL SOCIETY

Held in Hurd Memorial Hall, January 13, 1947

The Mechanism of Excretion of Ammonium Thiosulfate. DR. JOHN FRANKLIN and DR. JACQUES GENEST (*Department of Medicine, Johns Hopkins Hospital*).

Since the work of Gilman et al. in dogs and Newman et al. in man on the mechanism of excretion of the thiosulfate ion by the kidney showed that the thiosulfate ion is filtered by the glomerulus and not secreted or reabsorbed by the renal tubule, we have determined the fate of intravenously and orally administered ammonium thiosulfate in dogs. As it is an obligatorily excreted anion, we were interested in its effect on the pattern of electrolyte excretion, particularly in reference to the excretion of fixed base.

In dogs the intravenous administration of ammonium thiosulfate for short periods resulted in diuresis and rapid loss of sodium in the urine. Thiosulfate was recovered in the urine partly as sulfate (20–30 percent) and mainly as thiosulfate (70–80 percent). Oral administration of thiosulfate led to recovery of one-half as thiosulfate and one-half as sulfate in the urine. The loss of sodium was not so marked as from the intravenous administration due to the increased production of ammonia.

The continuous intravenous administration of ammonium thiosulfate resulted in excessive sodium excretion, renal failure and death with a serum electrolyte picture similar to that of Addison's disease; a very low serum sodium (125 Meq/Lt) and a high serum potassium (12 Meq/Lt). Analysis of the water content of this dog's muscle showed an increase to 89 percent water above a normal of 76 percent.

The potentiality of the drug as a diuretic and as a tool for interpreting the intricacies of the excretion pattern of electrolytes in clinical problems of salt and water balance was suggested.

DISCUSSION

Dr. Ephraim Shorr: What do you regard as the mechanism of the renal failure observed after continuous intravenous administration of ammonium thiosulfate?

Dr. John Franklin: We have no direct evidence as to the cause, but we feel that it was secondary to the circulatory failure associated with what we regard as terminal potassium intoxication. Darrow, Finch and others have demonstrated the serious cardiac effects of potassium poisoning. Cardiac standstill has been recognized as the immediate cause of death.

Dr. James Bordley: I would like to ask if you know exactly where the thiosulfate is converted to sulfate; whether that event occurs in the kidney or whether it occurs elsewhere in the body, because I should think the amount of sodium which is removed to some extent depends upon the place where that conversion occurred.

Dr. Franklin: I cannot answer that question, Dr. Bordley, since we have not studied that specific problem. Gilman has studied the volume distribution of thiosulfate and has observed that oxidation occurred for the most part during and immediately following injection. We feel that by the oral route, a major portion of the conversion takes place in the gastrointestinal tract. Sulfate excretion in the oral experiment accounted for approximately 50 percent of the recovered drug. We have extended Gilman's work by showing that the increased sulfate excretion accounts for that portion of the administered drug not recovered as thiosulfate. Gilman recovered some 70 to 80 percent of the administered drug, but did not account for the remaining 20 to 30 percent.

Dr. Richard Bing: I would like to ask whether you have seen any changes in renal plasma flow? It has been said that sodium thiosulfate does produce an increase in renal plasma flow and I wondered if you have seen similar changes in these experiments?

Dr. Franklin: We have not determined renal plasma flow in these experiments. However, the constancy of the thiosulfate clearance in humans despite high serum concentration after the single injection technique suggests that there was no significant vasodilatory effect.

Dr. John Luetscher: The chart of blood changes after intravenous ammonium thiosulfate showed a terminal fall in total serum proteins. I wonder if you have any comment on the fact that this is quite different from the usual rise in protein concentration in acute and chronic dehydration in man and experimental animals.

Dr. Franklin: I did not comment on that because we do not have a good explanation. There is one observation that can be made. The fall in total serum proteins occurred during the period of renal failure when the dog was in positive fluid balance. We can surmise that one of two things occurred. First, that the limit of transfer of water into the cell had been reached, or secondly, that in the face of decreased fluid output and continued infusion of ammonium thiosulfate, serum dilution was present. If you noticed, there was also a terminal fall in the hematocrit from 47 to 42 percent. I have no further explanation. Perhaps you have.

Dr. Luetscher: Did Dr. Zierler find any evidence of a transfer of protein into the cells?

Dr. Franklin: No, only the transfer of water into the cell was measured.

Dr. Shorr: I wonder whether the fall in blood volume was of sufficient magnitude to provide an explanation for renal failure similar to what which occurs in circulatory collapse from peripheral circulatory failure and secondary shock?

Dr. Franklin: I should imagine that decreased plasma volume played a role in the renal failure. We felt that potassium intoxication played the major role, however, because of the rapidity of exitus of this dog. Terminally, there was evidence of some serum dilution.

Hepatorenal Factors in Traumatic and Hemmorrhagic Shock. DR. EPHRAIM SHORR (*Cornell University Medical College, New York, N. Y.*).

Dr. Shorr described his technique of assaying vaso-excitor and vaso-depressor substances in the blood during various types of shock. The part played by the liver and kidneys in the formation and elimination of these materials was discussed. Failure of the normal mechanisms during prolonged shock may account for the "Irreversibility" of the late stages of shock.

DISCUSSION

Dr. James Bordley: Dr. Shorr, I wasn't quite clear on one point. You spoke of the relative oxygen requirements of the kidney and the liver as possibly explaining the fact that the kidney factor acted before the liver factor. Is the same thing true in vitro? Does the kidney factor appear at a lower oxygen tension than the liver factor?

Dr. Shorr: The kidney factor appears more readily than the liver factor. Of course, when we are dealing with slices in vitro I think we have to remember that we set up semi-artificial conditions. For example, the effect of a given concentration of oxygen will depend on the thickness of the tissue, so that kidney slices must be very thin. Liver slices can be thicker, and for that reasons it is a little difficult to be as arbitrary about the relative effects of reductions in oxygen tension on the appearance of the vaso-excitor factor. If we were to cut our kidney slices half as thin as we ordinarily do, then a greater reduction in oxygen tension would be required to produce a similar degree of anoxia, so that I think it is a little unsafe in view of the fact that we are dealing with the process of diffusion to be too dogmatic about relative reductions in oxygen tension which might be required by the kidney as compared to the liver.

Dr. Alfred Blalock: I have enjoyed this presentation by Dr. Shorr on this very important problem. He referred at the beginning of his paper to some of the earlier work that had been done on so-called toxic substances. As a result of the work of Cannon, Bayliss and others it was generally assumed shortly following the

first World War that the most important initiating factor in traumatic shock was the absorption of toxic products from an injured area. Studies in the 1930's cast a good deal of doubt on the results and the interpretations of results that had been given earlier. These studies showed that the local and regional loss of whole blood or plasma or both is the most important initiating factor in the vast majority of cases of traumatic shock. The liberal use of blood and plasma reduced tremendously the number of instances of shock that is seen in surgical patients. Unfortunately we still see an occasional patient who develops shock and who does not respond well to therapy. I think that such cases are seen more often on a medical service than on a surgical service. In fact, much remains to be learned about the pathogenesis of shock in many so-called medical disorders.

It is my impression that the pathogenesis of some instances of surgical or traumatic shock is fairly well understood if we limit the consideration to the early or so-called reversible stage. The question becomes much more complicated when we deal with the advanced or so-called irreversible stage. Many alterations have occurred at that time and there are probably many factors at work in causing the "irreversibility". It is on this stage of shock that Dr. Shorr's contributions are particularly valuable.

I think that one might raise a good many questions regarding the findings which Dr. Shorr has reported. For example, one might question the interpretation of results which are obtained by observing the capillaries of the meso-appendix. Even if it is agreed that one material is "vaso-depressor" and another is "vaso-excitor", there is no proof that the former is the cause of shock. The question can be raised as to whether the blood vessels of the rat's meso-appendix are a good indication of the reaction of the blood vessels of the rest of the body. After all, one is looking at only a few capillaries in a restricted area of the body.

Dr. Shorr reported experiments in which a tourniquet was applied to an extremity and this extremity was subsequently placed in a cast. The statement was made that no fluid could be lost into the extremity after the trouniquet was removed. It is true that a great quantity of fluid cannot be lost but I would suspect that there is some loss.

In two of the last slides which were shown by Dr. Shorr you will have noted that one of the initial features of the experiment was the removal of blood equal to 2½ to 3% of the body weight of the animal. Loss of blood in this amount will in some animals by itself alone cause shock and should certainly be taken into account when one is adding this procedure to some other method of causing shock.

The clinician should always keep in mind the maintaining of the blood volume at as nearly the normal level as possible. In this manner the vast majority of cases of shock can be prevented. This statement, however, is in no way meant to minimize the importance of studies such as Dr. Shorr has reported on the advanced stages of shock. It is to be hoped that such studies will result in one's being able to alter what is now regarded as irreversible shock to the type that can be effectively treated.

Dr. Shorr: I appreciate your comments, Dr. Blalock. First of all, as to the test. It is not something that is learned very rapidly. We take about six weeks to two months to train our technicians and work entirely by a code system. It has been this method, for example, by which out of a crude liver extract a material with consistent vaso-depressor activity has been fractionated, so that it had to be reasonably reliable to permit us to make that fractionation. I feel that objections to the test require us to provide, if possible, other pharmacological tests for the same material; and this we are attempting to do, so that it will be possible to duplicate the work without quite the same amount of apprenticeship to a difficult method.

Now as to the induction of shock in the animals whose limbs are taped, what I meant to say was that the amount of fluid loss in those limbs was so small as not under ordinary circumstances to produce shock. Actually those limbs were dry. However, in taping, unless casts are placed and even in the presence of casts, it is possible to lose a moderate amount of fluid, but I think that the extent of the fluid loss is much too small to be responsible for this sequence of events which leads to the profound and final circulatory failure. We have worried about just where the fluid goes and I think some experiments in eviscerated rabbits are helping us, as well as experiments in animals on low protein diets who have lost their inactivating system. I have a high respect for the capacity of the liver to take up blood. Shock can be induced in an eviscerated animal with the kidneys tied off, the liver intact, the portal vein tied off, and the hepatic artery furnishing the only supply to the liver; ligate the hepatic artery for a matter of 60 to 90 minutes for the formation of the vaso-depressor material, release it at the end of that time and flood the circulation with vaso-depressor material and the animal goes into circulatory collapse. Without the liver in those animals injection with vaso-depressor material will not cause them to go into shock. Our experiments would indicate that the liver is a very likely organ for pooling of blood and once the liver vessels are dominated by vaso-depressor material their sphincter arrangement is such as to allow them to pool just as well as the mesentery. While this is to some degree inferential, the congestion of the liver that is seen in late shock indicates that it does indeed take up blood. Now when one releases the tourniquet from the limb that has been placed in the cast and into which very little blood loss has taken place, one finds that the first thing one can observe is the release of the vaso-depressor material in quantities from those limbs and then it is taken up very quickly in the liver. We picked it up at the time when the liver inactivating system was entirely intact; whereas we know that if the liver had formed as much vaso-depressor material as that, the liver inactivating system would have been severely damaged. The liver had taken up the vaso-depressor material, which has led to pooling of blood in the liver and the reduction of blood volume.

I would like to point out what I think I may not have emphasized—that we believe that the fundamental factor which makes against recovery is the damage to the inactivating system. Unfortunately, shock of this character is associated with

renal impairment so that the renal excretory mechanism is of no help. But if the hepatic inactivating system is helped to liberate the blood stream of the vasodepressor material then transfusion should take hold. It may very well be that the management of shock through massive transfusions of whole blood which were given the latter part of the war may have achieved success by allowing sufficient time for survival of the patient during which there could be a regeneration of the liver enzyme system.

FEBRUARY 17, 1947

The Implications of Rh Incompatibility in Transfusion of Women. DR. LOUIS M. HELLMAN (*Department of Obstetrics*).

Nine Rh-negative women are presented who have received transfusions and who subsequently delivered children with erythroblastosis or developed antibodies which endangered their future childbearing careers. These nine women had five normal children prior to their transfusions and but two following. Both of the normal children after transfusion were Rh-negative and these women had heterozygous husbands. The patients with homozygous mates had no normal children after transfusion, and of the eight born to them, the expected survival rate of 50 percent occurred. The future childbearing of all of these women has been seriously jeopardized and particularly those with homozygous husbands. At least one of these latter women has requested and received sterilization for this reason.

It might well be argued that all but three of these patients had one or more pregnancies prior to the transfusion and that the pregnancy alone might well be sufficient to immunize them. Certainly this fact cannot be denied but it is interesting to note that a history of previous transfusion occurs in but two percent of our patients, while in the twenty-seven instances of erythroblastosis a history of transfusion was present in seven women or twenty-six percent. It is difficult to feel that transfusion does not play an important role in the production of erythroblastosis. With the increased use of transfusion the chances of unwittingly administering Rh-positive blood to Rh-negative females has definitely increased. It would appear that a high degree of negligence is present if transfusion is ever given to a female child or women in the childbearing age without first determining the Rh-compatibility. This is certainly the very minimum care that can be expected. For some time now no transfusion has been given in the Johns Hopkins Hospital to individuals of either sex without first determining the Rh-compatibility, except in cases of dire emergencies and then Rh-negative blood is always given.

Conclusions: Nine Rh-negative women are presented who gave a history of transfusion.

2. The probable damage to their future childbearing careers is pointed out.

3. The necessity of comparing Rh-compatibility prior to the transfusion of females is emphasized.

DISCUSSION

Dr. Eastman: I should like to make a few remarks supplementing and re-emphasizing what Dr. Hellman has said about the role which transfusions play in causing stillbirths and neonatal deaths. We are becoming increasingly impressed by the role that transfusions rather than repeated childbirths play in bringing about this entity. An Rh-negative woman married to an Rh-positive man can have baby after baby sometimes without any dire results because occasionally the Rh-positive cells do not find their way through the placenta and for this or other reasons she is not immunized. But when we have an Rh-negative mother married to an Rh-positive husband, and let there be a history of transfusion, we feel that the chances are about nine out of ten that she will get into difficulty and will have been rendered unfit for childbirth. Now Dr. Hellman has referred to the fact that after all this disease doesn't occur very often and perhaps we have exaggerated the importance of it. A week or so ago at one of the medical clinics I referred to the fact that today erythroblastosis is a commoner cause of stillbirth and neonatal death than syphilis. One or two of the group later raised some question about this and I looked it up and found that in the City of Chicago in the year 1944 the stillbirths and neonatal deaths due to erythroblastosis exceeded those due to syphilis. These tragedies which we see so frequently cause a great deal of heartache and mental anguish and I would go much further than Dr. Hellman and beg of you in the practice of medicine, surgery and pediatrics or any others who have to do with the transfusing of females, whether they be a few hours old or whether they be 45 years old, to consider that the chances are in the order of one to seven or eight that the transfusion is Rh incompatible. Accordingly, I urge you in these cases to do Rh cross-matching so that we here will be in a position to say that we are not responsible for this accident having occurred.

Dr. Janet Hardy: I would like to emphasize the importance of Dr. Hellman's paper from the point of view of the pediatrician. One cannot help being impressed by two things. Firstly, that these erythroblastotic babies whose mothers were sensitized by blood transfusions, tend to be terribly sick and present complicated problems of medical care; 50% of them die. Secondly, the majority of infants seen here with erythroblastosis during the past two years have been born to mothers sensitized by blood transfusions. Few have been born to mothers sensitized by previous pregnancies and these have all been mild, presenting no serious problems.

Physiological Studies in Congenital Heart Disease. DR. RICHARD BING, DR. L. R. VANDAM and DR. FRANK GRAY (*Department of Surgery*).

Dr. Bing's reports were published in the February and June issues of the BULLETIN.

DISCUSSION

Dr. James Bordley: I am sure that Dr. Bing is fully aware of the questionable validity of the assumptions upon which some of his conclusions are based. He and his associates deserve a great deal of credit for having undertaken this formidable job in spite of the theoretical objections which could be raised. They have had opportunity to make observations on an astonishingly large group of individuals suffering from what would usually be considered a rare cardiac disturbance. The correctness of many of their deductions has been verified by subsequent surgical intervention, and by repetition of observations after operations. Their experience clearly indicates that in spite of the questionable assumptions and theoretical objections, information can be obtained by their methods, which is of real practical value in deciding whether to operate on a patient who has multiple congenital cardiac defects. Quite apart from the findings in this particular group of cases, it should be pointed out that these methods can give practical assistance in arriving at an accurate diagnosis in less complicated types of cardiac defects. This was recently demonstrated to me in the case of a patient whom I referred to Dr. Bing. This young woman, aged 32 years, had led a perfectly normal life until the age of 24 when in the midst of a pregnancy she was found to have a loud cardiac murmur. Since then she had been treating herself as an invalid though she had few specific symptoms referrable to the heart. On physical examination, the heart was not enlarged but there was a loud murmur over the upper left chest and back, quite typical of the murmur of a patent ductus. Fluoroscopic and x-ray studies showed that the heart and great vessels were normal in size and there was no undue prominence of the conus or pulmonary vessels. We could not be certain that the patient had a large enough shunt from aorta to pulmonary artery to cause the vague symptoms of which she complained. Nor could we be certain that we were dealing with an uncomplicated patent ductus. Dr. Bing and his associates calculated from their observations that the output of the left ventricle was about twice that of the right ventricle. They found no evidence of any other cardiac defect. The conclusion was that the patient had a large shunt through the ductus, and this proved to be the case at operation. Dr. Blalock found that there was a patent ductus measuring more than one centimeter in diameter. The ductus was closed and all of the signs over the heart disappeared. Two months after operation the patient is now making very satisfactory progress. In this case, Dr. Bing's findings afforded great practical assistance in the proper evaluation of the patient's symptoms and gave us much greater confidence in discussing prognosis and treatment with the patient and her family.

Dr. Joseph Lilienthal: I think it is probably fair to say that Dr. Bing and his colleagues have had a unique experience in their opportunity to study so large a group of individuals who have been exposed to chronic anoxemia for months and years. Furthermore, they have had an opportunity to study them while the investigators themselves were under normal conditions in contrast to the studies

carried out at high altitude. For these reasons it would be of interest to learn what data have been observed which may throw light on the adjustments which these patients have made in response to chronic anoxemia. Dr. Bing intimated that there was a change in basal oxygen consumption. Would he be good enough to amplify on this interesting observation and any other data of this nature?

Dr. Bing: We don't have many data on the subject yet. A dissociation curve is being constructed in this laboratory by Dr. Spencer with the method of Dr. Lilienthal and Dr. Riley. We have found that the dissociation curve is not different from the normal curve; in other words, it does follow the curve which is considered as a standard curve. This indicates that hemoglobin is not different from normal hemoglobin.

Dr. Arnold Rich: May I ask Dr. Bing whether he found any cases in his studies that would indicate that the collateral circulation is equal to or greater than the pulmonary flow?

Dr. Bing: There are such cases in which the collateral circulation exceeds the volume of flow through the pulmonary artery. This is especially true in cases of complete pulmonary atresia. Drs. Blalock and Duncan have confirmed this.

Conversion of Normal into Malignant Cells in Vitro. DR. GEORGE O. GEY (*Department of Surgery*).

This study is concerned with a series of permanent alterations occurring in continuous cultures of normal rat mesenchyme cells and leading to the production of malignant cells. The strains studied include normal, altered normal, and malignant cell strains, all of autologous origin, which have been cultivated in roller tubes for eight and one-half years. These strains make it possible to make direct comparisons between a normal and a malignant strain derived therefrom. The data to date suggest that factors contributed by the culture medium, which is totally heterologous, are important and may play a role in the alterations observed. To date, no known extraneous carcinogenic agents have been found which might have brought about these conversions which occurred in stocks of normal cell strains. In this presentation some of the differences between the normal and the malignant autologous strains were discussed. Certain differences in morphology and activity were illustrated with lantern slides and phase-contrast motion pictures.

DISCUSSION

Dr. Wilton Earle (National Cancer Institute): I didn't intend to come here to talk, but just to hear this interesting work reported by Dr. Gey. I have been following Dr. Gey's work for quite a number of years, and meanwhile our own work has been carried on along very similar lines since in our work we have been treating normal mouse fibroblasts with the carcinogen 20-methylcholanthrene. In a num-

ber of ways our results parallel Dr. Gey's. In our studies we did get a great change in our treated cultures and a slight change in our untreated control cultures. The change in the control cultures was a slight one and the cells were just perceptibly altered morphologically from the normal, yet that strain of cells has given a greater percentage of sarcomas on injection than any of the cell strains deliberately treated with the carcinogen; one group of cultures of this strain gave us 100% sarcomas on injection. Sections of these sarcomas showed very slight anaplasia; metastases have not occurred, and while there was infiltration there was little cachexia. With cultures substantially more altered by deliberate addition of the carcinogen for a short interval of days, we obtained a smaller percentage of sarcomas produced on injection of the treated cells, but in sections of these sarcomas there was greater anaplasia and metastases did occur.

We do not yet know what agent or agency caused the limited degree of neoplastic change in the control cultures, but I think it very likely they received an accidental trace contamination with the same carcinogen. Leaving the behavior of these control cultures out of consideration, however, the results from our cultures indicate that the greater the exposure time of the cells to a constant concentration of carcinogen, the greater the change in the cells. Further, I think we can say that in our cultures at least, this change for any one culture seems reasonably uniform throughout the culture. In Dr. Gey's cultures it was most interesting that he found his changes occurring in small groups of cells; in our studies the cultures appeared to change as a whole.

We are just completing the tabulation of our data and I think we can now say that of the six strains of neoplastic cells we have originated in vitro, all from one parent strain of normal mouse fibroblasts, it now appears that three out of the six have preserved their altered morphology without further change for over four years since their removal from the carcinogen. Three strains do show some continued change, but certain recognizable characteristics peculiar to each other have persisted unchanged. This apparent continued change since removal from carcinogen may, however, be due in whole or in part to secondary cell selection of more hardy cells in propagation of the cultures. In the remaining three strains, however, there has been no further recognizable change in their morphology for over 4 years in culture. In these three strains, if we allow, generously, 5 days for the average intermitotic interval of their cells, it then appears that the cells of these strains have been able to stabilize their respective altered morphologies so that they have been preserved through more than 300 consecutive generations of cell mitosis since removal of the cells from carcinogen.